BCL-2 levels do not predict azathioprine treatment response in inflammatory bowel disease, but inhibition induces lymphocyte apoptosis and ameliorates colitis in mice

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Abstract: In inflammatory bowel disease (IBD), inflammation is sustained by an exaggerated response of lymphocytes. This results from enhanced expression of anti-apoptotic B cell lymphoma (BCL-2) and BCL-XL associated with a diminished turnover. Azathioprine (AZA) directly targets BCL-2 family-mediated apoptosis. We investigated whether the BCL-2 family expression pattern could be used to predict treatment response to AZA and determined whether BCL-2 inhibitor A-1211212 effectively diminishes lymphocytes and ameliorates inflammation in a model of colitis. BCL-2 family expression pattern was determined by next-generation sequencing (NGS). BCL-2 inhibitor was administered orally to Il10-/- mice. Haematological analyses were performed with an ADVIA 2120 and changes in immune cells were investigated using quantitative polymerase chain reaction (qPCR) and fluorescence activated cell sorter (FACS). We determined similar expression levels of BCL-2 family members in patients with remission and patients refractory to treatment, showing that BCL-2 family expression can not predict AZA treatment response. Expression was not correlated with the modified Truelove and Witts activity index (MTWAI). BCL-2 inhibitor initiated cell death in T cells from patients refractory to AZA and reduced lymphocyte count in Il10-/- mice. FACS revealed diminished CD8 T cells upon BCL-2 inhibitor in Il10-/- mice without influencing platelets. Tnf, Il1 , Ifn and Mcp-1 were decreased upon BCL-2 inhibitor. A-1211212 positively altered the colonic mucosa and ameliorated inflammation in mice. Pro-apoptotic BCL-2 inhibitor A-1211212 diminishes lymphocytes and ameliorates colitis in Il10-/- mice without inducing thrombocytopenia. BCL-2 inhibition could be a new therapy option for patients refractory to AZA.

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BCL-2 levels do not predict azathioprine treatment response in inflammatory bowel disease, but inhibition induces lymphocyte apoptosis and ameliorates colitis in mice

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Running head BCL-2 selective inhibition by A-1211212 ameliorates colitis

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Abbreviations: Azathioprine (AZA), Inflammatory bowel disease (IBD), intestinal epithelial cells (IECs), ulcerative colitis (UC), Crohn's disease (CD)

Abstract

Introduction: In inflammatory bowel disease (IBD), inflammation is sustained by an exaggerated response of lymphocytes. This results from enhanced expression of anti-apoptotic BCL-2 and BCL-XL associated with a diminished turnover. Azathioprine (AZA) directly targets BCL-2 family-mediated apoptosis. We investigated whether the *BCL-2* family expression pattern could be used to predict treatment response to AZA and determined whether BCL-2 inhibitor A-1211212 effectively diminishes lymphocytes and ameliorates inflammation in a model of colitis.

Methods: *BCL-2* family expression pattern was determined by next generation sequencing (NGS). BCL-2 inhibitor was administered orally to *Il-10*$^{-/-}$ mice. Haematological analyses were performed with an ADVIA 2120 and changes in immune cells were investigated using qPCR and FACS.

Results: We determined similar expression levels of *BCL-2* family members in patients with remission and patients refractory to treatment, showing that *BCL-2* family expression can not predict AZA treatment response. Expression was not correlated with the modified Truelove and Witts activity index (MTWAI). BCL-2 inhibitor initiated cell death in T cells from patients refractory to AZA and reduced lymphocyte count in *Il-10*$^{-/-}$. FACS revealed diminished CD8^{+} T cells upon BCL-2 inhibitor in *Il-10*$^{-/-}$ without influencing platelets. *Tnf*, *Il1β*, *Ifnγ* and *Mcp1* was decreased. A-1211212 positively altered the colonic mucosa and ameliorated inflammation in mice.

Conclusions: The expression pattern of *BCL-2* family genes does not predict AZA treatment response in IBD. Pro-apoptotic BCL-2 inhibitor A-1211212 diminishes lymphocytes and ameliorates colitis in *Il-10*$^{-/-}$ mice without inducing thrombocytopenia. BCL-2 inhibition could be a new therapy option for patients refractory to AZA.

INTRODUCTION

Inflammatory bowel disease (IBD) affects about one in a hundred and fifty people in the industrialized world. IBD is characterized by a chronic inflammation of the intestinal wall and comprises two main conditions, ulcerative colitis (UC) and Crohn's disease (CD). The inflammatory state in IBD is sustained by an exaggerated response of T-lymphocytes to luminal antigens, which is associated with an increased resistance to apoptosis in these cells (1). The B-cell lymphoma (BCL)-2 family plays a critical role in the control of immune responses by regulating the expansion and contraction of the activated lymphocyte population via apoptosis. Pro-survival BCL-2 family members (BCL-2, BCL-XL, BCL-ω, MCL-1 and A1) interact with pro-apoptotic BCL-2 family members (e.g. BAX and BAK from the BAX group, and BIM and BMF from the BH3-only group), and induce or abolish apoptosis. All BCL-2 family members share a common BH3 domain. The balance of interactions between BCL-2 family members determines the celullar lifespan. Most activated T cells die at the end of a T cell response, which coincides with decreased levels of BCL-2 *in vivo* (2, 3). In patients suffering from IBD, the lifespan of antigen-primed and activated T cells is extended, and an abnormal population of activated T cells is retained within the mucosal compartment (1, 4, 5). Enhanced expression of the anti-apoptotic BCL-2 is found in lamina propria lymphocytes from inflamed tissue in CD patients (4). These cells also present activation of the STAT-3 signalling pathway, which mediates the expression of anti-apoptotic *BCL*-2 and *BCL*-XL (6). Resistance of T cells to apoptotic signals is correlated with an increase in BCL-2 expression in CD patients (4). In contrast, apoptosis of anti-inflammatory regulatory T cells is reduced in IBD patients (7).

Medical therapies that regulate proliferation and contraction of lymphocyte populations are directly linked to the BCL-2 family-mediated apoptosis (8, 9). Azathioprine (AZA) and its metabolite 6-mercaptopurine (6-MP) are immunosuppressive agents that interfere with T cell proliferation via its metabolite 6-thioguaninnucleotide. AZA therapy reduces blood and lamina propria natural killer cells by promoting apoptosis (10). AZA supresses BCL-XL, thereby leading to apoptosis through the mitochondria-mediated apoptosis pathway (11). Likewise, other central therapeutic agents, such as sulfasalazine (12), glucocorticoids (13) and anti-TNF antibodies (14-16) are potent pro-apoptotic agents modulating the activity of BCL-2 family members.

Boosting the initiation of cell death in apoptosis-resistant lymphocytes could improve the success of medical therapy. A number of BH3 mimetics that directly antagonize BCL-2 proteins are available. ABT-737 is a potent inhibitor of BCL-2, BCL-XL and BCL-ω (17, 18), and disrupt the interactions of BCL-2 family proteins, resulting in apoptosis in lymphoma and other blood cancers (19). Because of its unfavorable pharmacologic properties, ABT-737 is not appropriate for clinical trials, while its orally bioavailable derivative navitoclax (ABT-263), which also inhibits BCL-2, BCL-XL and BCL-ω, is an experimental anti-cancer drug without off-target effects (20). Inhibition of BCL-XL may be responsible for the observed reduction of platelet lifespan and may also cause thrombocytopenia upon navitoclax treatment. To overcome side effects, BCL-2 selective BH3 mimetics were developed. Venetoclax (ABT-199, trade names Venclexta and Venclyxto, AbbVie) inhibits BCL-2 selectively, constituting the first substance from the chemical class of BCL-2 inhibitors approved by the FDA. Venetoclax is an oral drug for the treatment of chronic lymphocytic leukemia in those with a specific chromosomal abnormality. However, neutropenia has been reported as a side effect. A-1211212 (a close analog of venetoclax) is a BCL-2 selective orally

bioavailable inhibitor. Pro-apoptotic A-1211212 reduces the number of neutrophils in the circulation of rats without influencing the platelet counts (21).

BH3 mimetics that antagonize BCL-2 family proteins were initially developed for the treatment of cancer while exhibiting minimal toxicity toward normal cells. Additionally, a number of studies demonstrate that the BCL-2 inhibitor ABT-737 is also able to enhance apoptosis in pro-inflammatory/autoreactive lymphocytes escaping the physiological termination of their life cycle. Thus, ABT-737 is effective in treating animal models of autoimmune diseases (22), arthritis (23) and lupus (24). Furthermore, expression of the pro-apoptotic BIM, and the extent of its association with BCL-2, correlates with *in vivo* ABT-737 sensitivity (25). Recently, we showed that ABT-737 limits the persistence of lymphocytes in a mouse model of colitis (26). However, the amelioration of colitis was accompanied by lymphopenia.

In this study, we showed that the expression pattern of *BCL-2* family proteins does not predict the response to AZA treatment in IBD. Nonetheless, inhibition of BCL-2 initiated cell death in $CD4^+$ T cells from patients refractory to AZA. We also found that the BCL-2 inhibitor A-1211212 effectively diminished accumulated lymphocytes and ameliorated colitis without inducing thrombopenia in a murine model of spontaneous colitis. Regulating inappropriate survival of autoreactive lymphocytes through the inhibition of BCL-2 may provide a new therapeutic strategy in IBD.

MATERIALS AND METHODS

Ethical Considerations

We retrieved data from 48 patients (supplementary table 1) from the SIBDCS, a longitudinal, prospective, cohort study of Swiss residents diagnosed with IBD (27) approved by the local ethical committees (EK-1316). Animal experiments were approved by the Cantonal Comission for Animal Experiments (160/2014).

Next generation sequencing (NGS)

Blood samples were collected from three patients with sustained remission following AZA treatment, and three patients refractory to treatment (supplementary table 1). NGS was performed using total RNA that passed Tapestation (Agilent) quality control with a RIN > 8.3 (supplementary figure 1). Illumina NGS was performed by the Functional Genomics Center Zurich.

Isolation and stimulation of human CD4$^+$ T cells from whole blood

Blood samples were collected before and after treatment with AZA from 27 patients with sustained remission following AZA treatment, 21 patients refractory to treatment and eight healthy controls without treatment (supplementary table 1). Peripheral blood lymphocytes (PBLs) were isolated using Ficoll (Histopaque-1077, 10771-100ML Sigma) and resuspended in medium: RPMI-1640 (21875-034, Gibco), 2 mM L-glutamine, 100 U/ml each of penicillin and streptomycin, 50 mM β-mercaptoethanol and 10 % FCS. CD4$^+$ T cells were isolated using the Cell Isolation Kit (130-096-533, Miltenyi). For stimulation of CD4$^+$ T cells, a well plate was coated 24 h before the experiment with anti-CD3 (0.04 µg/ml, 130-093-387, Miltenyi). CD4$^+$ T cells were cultivated (37°C, 5% CO_2) during 5 days in media containing IL-2 (40 IU/ml, 130-097-743, Miltenyi) and anti-CD28 (1 µg/ml, 130-093-375, Miltenyi). ABT-737, -199, -263 (1 µM each, AbbVie), 6-MP (5 µM, 852678-1G-A, Sigma) or vehicle (DMSO, 41640, Sigma) .

Flow cytometry and mass cytometry

Apoptotic cells were determined by annexin V (ALX-209-252-T100, Enzo Life Science) and propidium iodide (PI, P4864, Sigma) staining. Annexin V$^-$ / PI$^-$ cells were considered alive, while early apoptotic cells displayed staining for annexin V but not for PI. Late apoptotic cells displayed staining for both, annexin V and PI. Fluorescence was measured using a BD FACSCanto II flow cytometer. CD4 was stained with anti-mouse antibody conjugated with APC-Cy7 (1:200, 552051, BD biosciences), CD8 with anti-mouse PerCp Cy5.5 (1:400, 45-0081-80, eBioscience), CD3 with anti-mouse antibody APC (1:200, 100236, BioLegend), B220 with anti-mouse Cy7 (1:200, 552772,

BD Pharmingen), F4/80 with anti-mouse FITC (1:200, 8940F, Cedarlane) and CD45 with anti-mouse PB (1:800, 103126, BioLegend). Aqua staining was performed to discriminate live and dead cells (1:1000, L34957, Thermo Fisher Scientific).

Mass cytometry was performed as previously described (26) applying the MaxPar Cell Surface Staining Protocol (DVS Sciences, Toronto, Canda) including cell-ID cisplatin reagent (201064, Fluidigm) for viability staining, surface antibody cocktail (201306, MaxPar Mouse Spleen/Lymph Node Phenotyping Panel Kit) and iridium intercalation solution (201192B, Fluidigm). Cells were acquired on the CyTOF 2 instrument (Fluidigm). The resulting data were analysed with FlowJo (Flowjo LLC; TreeStar, Inc., Ashland, OR, USA) and Cytobank software (Mountain View, CA, USA).

qPCR

Transcriptional expression of murine *Bim* (#Mm00437796_m1), *Bcl-2* (#Mm00477631_m1*), *Bcl-xl* (#Mm00437783_m1), *Bid* (#Mm00432073), *Tnf* (#Mm99999068_m1), *Il1β* (#Mm01336189_m1), *Ifnγ* (#Mm00801778_m1), *Mcp1* (Mm00441242_m1) and *Tgfβ* (Mm01178820_m1) was determined by TaqMan. Actin (#4352341E) or glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, #4352339E) was measured as endogenous control. For human samples, *BCL-2* (#Hs00608023_m1), *BCL-XL* (#Hs00236329_m1), *BMF* (Hs00372937_m1) and *BIM* (#Hs01083836_m1) were used. Actin (#4310881E) or *Gapdh* (#4326317E) was measured as endogenous control (Applied Biosystems, respectively). Relative mRNA expression was calculated using the ΔΔCt method. All samples were analyzed as triplicates.

Murine spontaneous colitis model and treatment with BCL-2 inhibitors

Wild type mice (Jackson), B.6129P2-Il10^{tm1Cgn}/J (*Il-10*$^{-/-}$, Jackson) and B.6129P2-Il10^{tm1Cgn}/J x B6.129-Bcl2l11$^{tm1.1Ast}$/J (*Il-10*$^{-/-}$ x *Bim*$^{-/-}$) mice weighing 25-30 g were used. A-1211212 (AbbVie, USA) was applied by oral gavage (p.o., 3, 10, 30, 100 mg/kg/day) dissolved in 30% polyethylene glycol 400, 60% phosal PG 50 and 10% ethanol. ABT-737 was injected intra-peritoneally (i.p., 50 mg/kg/day) dissolved in polyethylene glycol, Tween 80, dextrose solution and DMSO. Animals were sacrificed on day 14 of treatment.

Assessment of colonoscopy and histological score in mice

Animals were anesthetized with isoflurane and examined with the Tele Pack Pal 20043020 (Karl Storz Endoskope, Germany). Mice were scored according to the murine endoscopic index of colitis severity (MEICS) as previously described (28). For histological analysis, both colon and small bowel were resected and scored as described (29, 30).

Isolation and culture of murine primary cells

For hematological analyses, murine whole blood was collected in EDTA-treated tubes (367525, BD vacutainer). Analyses were performed with an ADVIA 2120 flow cytometer (Siemens). For mass cytometry of murine PBLs, whole blood was collected in EDTA-treated collection tubes. ACK lysing buffer (1,5 M NH4Cl, 100 mM KHCO3, 10 mM Triplex111, ph 7.2) was used.

Splenocytes were isolated using the gentleMACS octo dissociator (130-095-937, Miltenyi, program m_spleen 03) and gentleMACS C tubes (130-093-237, Miltenyi) containing 10 ml ACK lysising buffer. Splenocytes were passed over a 70 µm mesh filter.

For the isolation of *lamina propria* mononuclear cells (LPMNCs), 10 cm of the small bowel was resected and cut in small pieces. 5 ml of a digestive enzyme complex containing Collagenase V (C9263-1G, Sigma), Collagenase D (11088882001, Roche), Dispase (17105-041, Gibco), DNase (10104159001, Roche) and RPMI medium (21875-034, Gibco) with 10% FCS was incubated with the small bowel for 30 min at 37°C. Suspension was vortexed and passed through a 70 µm mesh filter.

Murine $CD4^+CD62L^+$ cells from spleen were isolated with the T Cell Isolation Kit II (130-093-227, Miltenyi). For cell culture, a well plate was coated 24 h before the experiment with anti-CD3 (2 µg/ml, 130-092-973, Miltenyi). Cells were cultivated in media containing IL-2 (4 ng/ml, 402-ML-020/CF, R&D) and anti-CD28 (2 µg/ml, 130-093-182,

Miltenyi). ABT-737 (1 µM – 1 x 10^{-4} µM) and 6-MP (5 µM - 5 x 10^{-4} µM, final concentration each) or vehicle was added for 5 days (37°C, 5% CO_2).

MPO activity assay

Both colon and small bowel specimens were homogenized in 50 mM phosphate buffer with 0.5% hexadecyltrimethylammonium bromide (H-5882, Sigma) with the gentleMACS octo dissociator (130-095-937, Miltenyi, program mpo). After three freeze-and-thaw cycles, homogenates were centrifuged for 2 min at maximum speed. 20 ml of the supernatant were transferred to a 96-well plate in duplicate and mixed with 280 µl of 0.02% dianisidine (in 50 mM phosphate buffer and 0.0005% H_2O_2, D-3252, Sigma). Absorbance was measured either after 20 min, if samples were from *Il-10*$^{-/-}$ mice or after 60 min, if samples were from WT mice, at 460 nm. Protein concentration of the supernatant was determined by the BCA Protein Assay Kit (23252, Thermo Fisher Scientific). MPO activity was calculated as mean absorbance (460 nm)/incubation time/protein content.

Statistical analyses

All Pairwise Multiple Comparison Procedures (Dunn's Method): Kruskal-Wallis One Way Analysis of Variance on Ranks or a non-parametric t test was applied as indicated in the figure legends. Differences were considered significant at $p < 0.05$, highly significant at $p < 0.01$ and $p < 0.001$.

RESULTS

BCL-2 family expression does not predict AZA treatment response in IBD

First, we determined whether the mRNA expression pattern of BCL-2 family members could be used to predict the response to AZA treatment in IBD patients. For NGS analysis, blood samples were collected from patients with sustained remission (successfully treated patients, n = 3) or refractory to treatment (primary non-responders, n = 3, supplementary table 1). Following NGS analysis, we determined similar expression of 36 members of the *BCL-2*-family in patients with remission compared to patients refractory to treatment ($p > 0.05$ each, table 1). To confirm the results from NGS, we performed qPCR from 23 patients in remission, 18 refractory patients and six healthy controls. For qPCR, blood samples were collected before AZA medication therapy and after the outcome. We determined similar expression levels of *BCL-XL*, *BCL-2*, *BMF and BIM* in patients before AZA medication therapy with remission compared to patients refractory to treatment (supplemetary figures 2 - 5). mRNA expression was not correlated with the MTWAI and did not change over time, indicating that the expression of *BCL-2* family members can not predict AZA treatment response in IBD.

BCL-2 family inhibitors, but not 6-MP, induce T cell apoptosis in patients treated with AZA

Next, we determined whether the reduction of lymphocyte counts in IBD patients upon AZA treatment is increased by BCL-2 family inhibitors *in vitro*. Flow cytometry was performed to quantify the number of viable and apoptotic $CD4^+$ T cells upon treatment with the AZA metabolite 6-MP (5 µM) and the BCL-2 family inhibitor ABT-737 (1 µM)

applied for five days. In healthy controls, the relative number of early and late apoptotic cells was significantly increased upon 6-MP treatment *in vitro* compared to vehicle (38.1 ± 5.7 % vs. 22.5 ± 1.8 %, $ p < 0.039, figure 1A). The number of apoptotic cells was also significantly increased upon ABT-737 treatment (40.5 ± 7.2 %, $ p < 0.028, figure 1A) and the combined treatment with 6-MP and ABT-737 compared to vehicle (56.3 ± 17.1 %, * p < 0.05, figure 1A). The number of apoptotic $CD4^+$ T cells was also significantly increased upon combined treatment compared to 6-MP and ABT-737 treatment alone ($ p < 0.023 and $ p < 0.032, respectively, figure 1A).

In contrast, the relative number of early and late apoptotic $CD4^+$ T cells from IBD patients treated with AZA remained unchanged upon 6-MP stimulation compared to controls (21.1 ± 0.7 % vs. 25.6 ± 7.4 %, figure 1A). The number of apoptotic cells was significantly increased upon ABT-737 treatment (35.3 ± 8.1 %, $$ p < 0.040, figure 1A) and the combined treatment (36.7 ± 6.3 %, * p < 0.05, figure 1A) compared to vehicle. The number of apoptotic $CD4^+$ T cells was significantly increased upon combined treatment compared to 6-MP treatment alone ($$ p < 0.045, figure 1A).

The number of apoptotic $CD4^+$ T cells following 6-MP treatment was significantly increased in healthy controls compared to IBD patients treated with AZA (# p = 0.004, t-test, figure 1A) and the combined treatment with 6-MP and ABT-737 (# p = 0.011, t-test, figure 1A). The ratio between apoptotic and non-apoptotic $CD4^+$ T cells in the presence of 6-MP or vehicle was significantly decreased in IBD patients receiving AZA compared to healthy controls (1.21 ± 0.32 % vs. 1.69 ± 0.28 %, n = 9 and 6, respectively, * p < 0.05, supplementary figure 6A).

Next, we analyzed the effects of these agents on the expression of *BCL-2* family members. We determined a significant decrease in *BCL-XL* expression in $CD4^+$ T cells from healthy controls following 6-MP stimulation compared to vehicle (0.69 ± 0.12 vs. 1.00 ± 0.02, n = 3, respectively, p = 0.01, supplementary figure 6B). In $CD4^+$ T cells

isolated from IBD patients treated with AZA at time of medical examination, *BCL-XL* gene expression remained unchanged upon 6-MP treatment *in vitro* after five days compared to vehicle (1.00 ± 0.16 vs. 1.00 ± 0.00, n = 8 and 7, respectively, figure 1B). *BCL-XL* was decreased upon treatment with the BCL-2 family inhibitors ABT-737, ABT-199 and ABT-263 (0.62.3 ± 0.29, 0.80.3 ± 0.14, n = 8, and 0.62.3 ± 0.25, n = 5; respectively, figure 1B) with a significant difference for ABT-737 and ABT-263 (* $p < 0.05$ each, figure 1B). *BCL-XL* was also significantly decreased upon combined treatment of 6-MP and ABT-263 (0.59 ± 0.19, n = 5, * $p < 0.05$, figure 1B). No significant difference was determined between ABT-263 and co-treatments. Similarly, *BCL-2* remained unchanged upon 6-MP stimulation, but was decreased in the presence of the BCL-2 inhibitors (supplementary figure 6C, not significant).

BCL-2 family inhibitors initiate cell death in CD4$^+$ T cells from IBD patients refractory to AZA

Next, we determined whether the amount of CD4$^+$ T cells from IBD patients refractory to AZA can be further decreased upon combined treatment with 6-MP and the BCL-2 family inhibitor ABT-737. Blood was drawn from three IBD patients treated with AZA at time of medical examination, while a considerable resistance to treatment was indicated (supplementary table 1). In CD4$^+$ T cells from three IBD patients refractory to AZA, the number of early and late apoptotic cells was increased upon 6-MP treatment compared to vehicle (36.6 ± 14.1 % vs. 23.2 ± 12.6 %, n = 3, respectively, figure 2). The number of apoptotic CD4$^+$ T cells was further increased upon treatment with ABT-737 (61.3 ± 21.8 %, n = 3, figure 2) or the combined treatment (68.1 ± 14.6 %, n = 3,

p = 0.055, figure 2), suggesting that inhibition of BCL-2 initiated cell death in $CD4^+$ T cells from patients refractory to AZA.

Additionally, we determined whether cell survival can be also decreased upon combined treatment in murine $CD4^+CD62L^+$ splenocytes. Cell death also increased in a dose-dependent manner in splenocytes upon 6-MP treatment (supplementary figure 7A). As 6-MP was less effective in cells isolated from Balb/C mice compared to B6 mice, we determined *Bcl-XL* in $CD4^+CD62L^+$ splenocytes and found increased expression in Balb/C mice compared to B6 mice (p = 0.057, t-test, supplementary figure 7B). Cells isolated from Balb/C mice and B6 mice were used for combined treatment experiments. In Balb/C, apoptosis in splenocytes was further increased upon the combined treatment with 6-MP and ABT-737 compared to treatment with a single compound or vehicle (supplementary figure 7C), confirming the results obtained with human T cells. In B6, apoptosis in splenocytes upon 6-MP or combined treatment was significantly increased compared to vehicle (* $p< 0.05$, SEM, supplementary figure 7C).

A-1211212 is detected in blood and intestine following oral gavage in mice

The broad range BCL-2 inhibitor ABT-737, which inhibits BCL-2, BCL-XL and BCL-ω, triggers undesirable side effects, including lymphopenia. To reduce adverse side effects, we determined the impact of the BCL-2 specific inhibitor A-1211212 on mucosal inflammation using the *Il-10*$^{-/-}$ mouse model of spontaneous colitis. A calculated dose of 3, 10, 30 and 100 mg/kg/day was applied by oral gavage for 14 days. Peripheral blood, intestinal epithelial cells (IECs) and whole colon tissue of mice were harvested to confirm the accumulation of A-1211212 in these samples. Following 14 days of treatment, mass spectrometric (MS) analysis detected A-1211212 in sera in a dose-dependent manner (0.01 ± 0.00, 0.29 ± 0.09, 0.51 ± 0.24, 0.81 ± 0.13, 1.69 ± 0.01 µg/mL for vehicle, 3, 10, 30 and 100 mg/kg/day, n = 2, respectively). MS

analysis also detected A-1211212 in intestinal mucosa in a dose-dependent manner (1.7, 0.3, 20.4, 42.1 µg/g for vehicle, 3, 30 and 100 mg/kg/day, n = 1, respectively). Less A-1211212 was detected in IECs (< 0.1, 0.3, 1.1, 1.2 µg/g for vehicle, 3, 10 and 30 mg/kg/day, n = 1, respectively).

A-1211212 ameliorates spontaneous colitis

Next, we determined the influence of A-1211212 and the broad range inhibitor ABT-737 on mucosal inflammation. Externally, *Il-10*$^{-/-}$ mice showed a clearly visible rectal prolapse at the beginning of the experiment that was associated with cryptitis and a massive lymphocyte influx into the intestinal mucosa as determined by H&E staining (supplementary figure 8). Macroscopic mucosal damage was assessed by mini-endoscopy. Mice received either A-1211212 or vehicle, or ABT-737 or vehicle over 14 days. Wild type mice showed unchanged MEICS regardless of treatment (figure 3B). *Il-10*$^{-/-}$ mice receiving vehicle displayed an opaque mucosa and altered vascular pattern on day 14 (MEICS 4.6 ± 0.7 and 6.0 ± 2.12, A-1211212 and ABT-737, n = 8 and 6 respectively, figure 3B). Prominent thickening of the colon and thin feces were observed and the diseased regions presented mucosal bleeding frequently. During treatment with both A-1211212 and ABT-737, inflammation was ameliorated compared to vehicle-receiving mice. *Il-10*$^{-/-}$ mice treated with both A-1211212 and ABT-737 had a transparent mucosa with a regular vascular pattern and solid feces were visible (MEICS 1.3 ± 0.6 and 3.7 ± 1.3, for 10mg/kg/day A-1211212 and ABT-737, n = 5 and 6, respectively, figure 3B).

Microscopic mucosal damage was assessed by histological score. Mice received either A-1211212 (3, 10, 30, 100 mg/kg/day) or vehicle, or ABT-737 (50 mg/kg/day),

or vehicle over 14 days. Wild type mice showed unchanged histological score regardless of treatment (figure 4B). The histological score of *Il-10*$^{-/-}$ mice upon treatment with A-1211212 or ABT-737 was significantly lower compared to vehicle-treated mice in small bowel (2.17 ± 0.76 and 3.84 ± 0.28, for 30 mg/kg/day A-1211212 and vehicle, n=7 and 10, respectively, *** $p < 0.001$, figure 4B) and colon (2.36 ± 0.48 and 4.17 ± 0.54, for 30 mg/kg/day A-1211212 and vehicle, n=7 and 10, respectively ** $p < 0.01$, supplementary figure 9). *Il-10*$^{-/-}$ mice also presented less lymphocyte influx into the thickened lamina propria (white arrows, figure 4A). In summary, *BCL-2* inhibitors positively altered the colonic mucosa at both macroscopic and microscopic level in the *Il-10*$^{-/-}$ model of spontaneous colitis.

To quantify the formation of reactive oxygen species, we performed MPO activity assay. In contrast to WT mice, *Il10*$^{-/-}$ mice showed considerably enhanced MPO activity in both small bowel and colon (figure 5A and B). Compared to vehicle-treated mice, A-1211212 treatment decreased MPO activity in a dose-dependent manner in both small bowel and colon in *Il-10*$^{-/-}$ mice (47.08 ± 28.52 and 162.56 ± 141.25, for 100 mg/kg/day A-1211212 and vehicle, n=8 and 8, respectively, * $p < 0.05$, figure 5A).

We determined gene expression of pro-inflammatory cytokines in whole mucosal tissue in WT and *Il-10*$^{-/-}$ mice upon A-1211212 treatment. In WT, *Tnf, Il1β, Ifnγ, Mcp1* and *Tgfβ* gene expression was not regulated in a dose-dependent manner (not shown). Next, we determined whether the ameliorated colitis in *Il-10*$^{-/-}$ mice upon A-1211212 was associated with a decrease in pro-inflammatory cytokines. At day 14, *Tnf* gene expression was significantly decreased upon administration of 30 mg/kg/day A-1211212 compared to vehicle (0.46 ± 0.21 vs. 1.00 ± 0.24, n=7 and 8, respectively, * $p < 0.05$, figure 6A). *Il1β* was significantly decreased upon treatment with 10 mg/kg/day A-1211212 compared to vehicle (0.41 ± 0.23 vs. 1.00 ± 0.30, n=7 and 8, respectively, * $p < 0.05$, figure 6A). *Ifnγ* was significantly decreased upon treatment with 100

mg/kg/day A-1211212 compared to vehicle (0.57 ± 0.19 vs. 1.00 ± 0.15, n=5 and 8, respectively, * $p < 0.05$, figure 6A). *Mcp1* was significantly decreased upon treatment with 100 mg/kg/day A-1211212 compared to vehicle (0.43 ± 0.25 vs. 1.00 ± 0.63, n=7 and 10, respectively, * $p < 0.05$, figure 6A). In contrast, *Tgfβ* increased in a dose-dependent manner upon treatment with A-1211212 compared to vehicle (2.86 ± 2.92 vs. 1.00 ± 0.29, for 100 mg/kg/day A-1211212 and vehicle, n=9 and 10, respectively, figure 6B).

A-1211212 shows platelet-sparing features, but shifts lymphocyte populations *in vivo*

Hematological analyses were performed in wild type mice and in the *Il-10*$^{-/-}$ model of spontaneous colitis to determine whether the increase in number of apoptotic cells upon A-1211212 treatment was associated with a shift in cell populations. Erythrocyte fraction was not affected in wild type mice and *Il-10*$^{-/-}$ mice after 14 days of p.o. gavage of A-1211212 and i.p. injection of ABT-737, when compared to vehicle-receiving controls (table 2). The platelet fraction remained unchanged upon A-1211212 treatment, but was significantly decreased following ABT-737 administration when compared to vehicle-receiving controls (** $p = 0.005$, t-test, table 2). The lymphocyte fraction was significantly decreased in wild type and *Il-10*$^{-/-}$ mice after 14 daily administration of 100 mg/kg/d A-1211212 or 50 mg/kg ABT-737, when compared to vehicle-receiving controls (* $p < 0.05$, ANOVA, table 2). The absolute number of lymphocytes was significantly decreased in wild type mice upon administration of 100 mg/kg/d A-1211212 compared to controls ($0.39 \pm 0.04 \times 10^3$ cells/µL, $n = 5$ vs. $2.23 \pm 0.80 \times 10^3$ cells/µL, $n = 5$, respectively, t-test, ** $p = 0.008$). The absolute number of lymphocytes was also significantly decreased in *Il-10*$^{-/-}$ mice upon administration of 100 mg/kg/d A-1211212 compared to controls ($0.41 \pm 0.31 \times 10^3$ cells/µL, $n = 6$ vs. $1.80 \pm 0.99 \times 10^3$ cells/µL, $n = 8$, respectively, t-test, ** $p = 0.003$) and upon administration of 50 mg/kg/d ABT-737 compared to controls ($0.49 \pm 0.08 \times 10^3$ cells/µL, $n = 3$ vs. $1.17 \pm 0.40 \times 10^3$ cells/µL, $n = 3$, respectively, t-test, * $p < 0.05$). As inhibition of BCL-2 by ABT-737 is mediated by displacing the pro-apoptotic factor BIM, we also applied ABT-737 to *Il-10*$^{-/-}$ x *Bim*$^{-/-}$ mice. The fraction of lymphocytes in these mice was unaltered upon ABT-737 treatment (table 2) and the absolute number of lymphocytes remained unchanged, confirming that BIM is required for the protective effect of ABT-737.

The monocyte fraction was decreased in *Il-10*$^{-/-}$ mice after 14 daily administration of A-1211212 or ABT-737, when compared to vehicle-receiving controls (table 2). The granulocyte fraction was increased in *Il-10*$^{-/-}$ mice after 14 daily administration of A-1211212, and was significantly increased upon ABT-737 treatment (** $p = 0.002$, t-test, table 2). In *Il-10*$^{-/-}$ x *Bim*$^{-/-}$ mice, the fraction of granulocytes was unaltered upon ABT-737 administration and the absolute number of granulocytes remained unchanged.

The BCL-2 inhibitor significantly decreases the amount of CD8$^+$ T cells

CD4$^+$ and CD8$^+$ splenocytes from wild type and colitic *Il-10*$^{-/-}$ mice were analyzed by flow cytometry. In WT mice, a significant decrease in CD8$^+$ T cells following treatment with A-1211212 compared to vehicle-receiving controls was determined (6.5 ± 1.9 % vs. 14.5 ± 4.3 %, for 30 mg/kg/day A-1211212 and vehicle, n=4 and 7, respectively, ** $p < 0.01$, ANOVA, figure 7). Similarily to the results obtained from WT mice, we determined a significant decrease in CD8$^+$ T cells following treatment with A-1211212 compared to vehicle-receiving controls (4.0 ± 1.3 % vs. 11.7 ± 3.0 %, for 30 mg/kg/day A-1211212 and vehicle, n=5 and 5, respectively, ** $p < 0.01$, ANOVA, figure 7).

Additionally, we determined the CD8$^+$ T cell population in PBL in an individual measurement by mass cytometry. Confirmative to flow cytometry, the proportion of CD8$^+$ T cells was decreased upon A-1211212 (supplementary figure 10A and B). The proportion of central memory T cells was decreased upon A-1211212 treatment compared to vehicle-receiving controls (CD45$^+$, CD19$^-$, B220$^-$,TCRβ$^+$, CD3$^+$, CD8$^+$, CD44$^+$ CD62L$^+$, supplementary figure 10C).

DISCUSSION

In accordance with previous reports (11), our results show that the expression pattern of *BCL-2* family members in $CD4^+$ T cells is changed upon treatment with the AZA metabolite 6-MP. Nonetheless, transcriptome analysis using NGS indicates that the response to AZA treatment in IBD patients cannot be estimated from mRNA expression levels of *BCL-2* family members. Although refractory patients and patients in remission were carefully chosen for this study, treatment duration, medication therapy prior to the study, and co-medication during AZA treatment was variable for the benefit of patients. However, given the highly similar expression levels of *BCL-2* family members in both groups over time, a predictive value seems unlikely.

6-MP therapy is unable to rescue IBD patients resistant to AZA treatment, evidencing a pressing need for alternative therapies limiting lymphocyte lifespan in these patients. We could confirm previous results that 6-MP is reducing the number of $CD4^+$ lymphocytes *in vitro* in healthy controls not receiving AZA prior to blood collection. In contrast, reduction is limited in IBD patients already receiving AZA. It is well established that dysregulated apoptosis of activated lymphocytes driven by increased anti-apoptotic BCL-2 and BCL-XL contributes to the pathogenesis of IBD (1, 4, 6, 31, 32). Consequently, our results show that the BCL-2 inhibitors ABT-737, ABT-199 and ABT-263 limit the persistence of human $CD4^+$ lymphocytes in IBD patients receiving AZA prior to blood collection. Further, ABT-737 was shown to limit the persistence of human $CD4^+$ lymphocytes in IBD patients refractory to AZA treatment. Similar results were obtained in previous studies using different BCL-2 inhibitors in various inflammatory conditions characterized by the uncontrolled proliferation of lymphocytes, BCL-2 inhibitors were efficacious in reducing accumulated lymphocytes in murine models of autoimmune diseases (22), arthritis (23) and lupus (24), and suppressed allogeneic T-

and B-cell responses after skin transplantation *in vitro* and *in vivo* (33). A recent report assessing the effects of BCL-2 inhibitors in steroid-insensitive airway inflammation showed that BCL-2 inhibitors ameliorate corticosteroid-resistant airway inflammation (34). Increased BCL-2 was found to be responsible for the persistence of granulocytes in bronchoalveolar lavage fluid after allergic challenge. Notably, BCL-2 inhibitors were more efficient than steroids in inducing granulocyte apoptosis in cells from patients with severe asthma (34).

In our previous work, we showed that the broad range BCL-2 inhibitor ABT-737 ameliorates inflammation in both the murine model of DSS-induced acute colitis and the *Il-10*$^{-/-}$ model of spontaneous colitis (26). However, this effect on inflammation was accompanied by lymphopenia due to ABT-737-mediated inhibition of BCL-2, BCL-XL and BCL-ω (17, 18). Inhibition of BCL-XL may be responsible for the reduction of platelet lifespan and the observed thrombocytopenia. As a consequence, in the present study we administered the newly developed BCL-2 selective pro-apoptotic compound A-1211212 to the *Il-10*$^{-/-}$ model of spontaneous colitis to avoid adverse side effects. A-1211212 is a BH3-only mimetic that shows a potent action against various transformed cells, while exhibiting minimal toxicity toward normal cells. Similarly to ABT-737, the specific BCL-2 inhibitor A-1211212 was detected in peripheral blood, colon tissue and IECs following oral gavage. In contrast to ABT-737, we did not observe thrombocytopenia upon A-1211212 treatment. However, similarly to ABT-737, A-1211212 diminished proportions of $CD8^+$ and central memory T cells accompanied by a decrease in *Tnf, Ifnγ, Mcp1* and *Il1β* gene expression in A-1211212-treated mice. Naïve T cells survival is mediated by MCL-1 and BCL-2 (35). Consequently, $CD4^+$ naïve T cells escape apoptosis in the presence of A-1211212, which is not inhibiting

MCL-1. A-1211212 positively alters the colonic mucosa at both macroscopic and microscopic level, while ameliorating intestinal inflammation in the *Il-10*$^{-/-}$ model of spontaneous colitis, as shown by MEICS and histological score. Inactivation of BCL-2 is key for BIM to directly bind to pro-apoptotic BAX and BAK in order to initiate apoptosis (36). As inhibition of BCL-2 is also mediated by BIM, we administered A-1211212 to *Il-10*$^{-/-}$ x *Bim*$^{-/-}$ mice upon spontaneous colitis. Interestingly, colitis in mice lacking *Bim* was not ameliorated by A-1211212 treatment confirming the crucial role of this factor in the pharmacological inhibition of BCL-2.

Adjusting the balance between activated and regulatory lymphocyte populations through the inhibition of autoreactive lymphocytes may represent a promising novel therapeutic strategy for CD patients. Our results point to a potential use of the BCL-2 selective, platelet-sparing inhibitor A-1211212 as a new therapeutic drug for the treatment of intestinal inflammation, particularly for IBD patients refractory to AZA treatment.

Disclosure

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Titles and legends to figures

Figure 1: **6-MP-mediated apoptosis is abolished in CD4$^+$ T cells from IBD patients upon AZA at time of medical examination.** Flow cytometry and qPCR. (A) PI/annexin V staining of human CD4$^+$ T cells from healthy controls and IBD patients upon AZA at time of medical examination. ANOVA on Ranks, All Pairwise Multiple Comparison Procedures (Dunn's Method), n as indicated, error bars = SD, * $p < 0.05$. $ = Statistics considering healthy controls. $$ = Statistics considering patients. # = t-test, normality test (Shapiro Wilk) and equal variance test passed. (B) *BCL-XL* qPCR. ANOVA, All Pairwise Multiple Comparison Procedures (Dunn's Method), error bars = SD, * $p < 0.05$.

Figure 2: **Inhibition of BCL-2 initiates cell death in CD4$^+$ T cells from IBD patients refractory to AZA.** Flow cytometry from three IBD patients upon AZA treatment at time of medical examination, while a considerable deterioration to treatment was indicated.

Figure 3: **A-1211212 and ABT-737 ameliorate colitis in *Il-10*$^{-/-}$ mice.** (A) Miniendoscopy was performed with a limited number of mice: 23 WT mice and 38 *Il-10*$^{-/-}$ mice, (B) MEICS upon A-1211212 or ABT-737 treatment in WT mice and *Il-10*$^{-/-}$ mice suffering from spontaneous colitis following 14 days of treatment, n as indicated, error bars = SD. One Way ANOVA, All Pairwise Multiple Comparison Procedures (Holm-Sidak Method) for A-1211212 and non-parametric t test for ABT-737, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: **A-1211212 and ABT-737 ameliorate colitis in *Il-10*$^{-/-}$ mice.** (A) H&E, (B) histological score from small bowel of 23 WT mice and 48 *Il-10*$^{-/-}$ mice suffering from spontaneous colitis following 14 days of treatment with A-1211212. Arrows indicate influx of lymphocytes, error bars = SD. One Way ANOVA, All Pairwise Multiple Comparison Procedures (Holm-Sidak Method) for A-1211212 and non-parametric t test for ABT-737, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5: **A-1211212 decreases MPO in *Il-10*$^{-/-}$ mice in a dose dependent manner.** MPO assay using small bowel (A) and colon tissue samples (B) from 23 WT mice and 32 *Il-10*$^{-/-}$ mice suffering from spontaneous colitis following 14 days of treatment with A-1211212, error bars = SD. One Way ANOVA, All Pairwise Multiple Comparison Procedures (Dunn's Method), * $p < 0.05$.

Figure 6: **A-1211212 decreases inflammatory cytokine mRNA expression in *Il-10*$^{-/-}$ mice.** qPCR from small bowel. (A) *Tnf*, *Il1β*, *Ifnγ* and *Mcp1*, (B) *Tgfβ* mRNA expression in *Il-10*$^{-/-}$ mice suffering from spontaneous colitis following 14 days of treatment with A-1211212, n as indicated, error bars = SD. One Way ANOVA, All Pairwise Multiple Comparison Procedures (Dunn's Method) for *Tnf* and (Holm-Sidak Method) for *Il1β*, *Ifnγ* and *Mcp1*, * $p < 0.05$.

Figure 7: **A-1211212 decreases CD8$^+$ T cell population in splenocytes in WT and *Il-10*$^{-/-}$ mice.** Flow cytometry analysis of 23 WT and 26 *Il-10*$^{-/-}$ mice suffering from spontaneous colitis following 14 days of treatment with A-121121. (A) Representative images of flow cytometry analysis, n = total 49. Doublets discrimination performed, viable and dead cells included. Cells shown were gated to CD45$^+$ and CD8$^+$ or CD4$^+$ splenocytes cells, (B) Dose-dependent decrease of CD8$^+$ splenocytes in WT and *Il10*$^{-/-}$ mice upon A-1211212 treatment compared to vehicle as determined by flow cytometry, error bars = SD. One Way ANOVA, All Pairwise Multiple Comparison Procedures (Dunn's Method), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1

Gene name	primary non-response	successful	P value
BAD	174 ± 18	162 ± 11	P = 0.406
BAK1	487 ± 150	526 ± 50	P = 0.346
BAX	996 ± 76	954 ± 17	P = 0.396
BCL10	1213 ± 669	701 ± 185	P = 0.270
BCL11A	155 ± 86	187 ± 50	P = 0.612
BCL11B	1026 ± 562	2199 ± 1612	P = 0.300
BCL11B	1026 ± 562	2199 ± 1612	P = 0.300
BCL2	1164 ± 448	1800 ± 504	P = 0.178
BCL2A1	6226 ± 4276	4750 ± 2766	P = 0.642
BCL2L10	0.00 ± 0.00	0.80 ± 0.71	P = 0.121
BCL2L12	80 ± 27	117 ± 23	P = 0.138
BCL2L13	822 ± 38	864 ± 42	P = 0.271
BCL2L14	3.99 ± 0.66	3.96 ± 3.47	P = 0.991
BCL2L15	27 ± 4	27 ± 8	P = 0.895
BCL3	3775 ± 2072	2452 ± 466	P = 0.341
BCL6	43067 ± 28035	21750 ± 13060	P = 0.299
BCL6B	0.36 ± 0.62	1.53 ± 1.62	P = 0.309
BCL7A	85 ± 65	98 ± 72	P = 0.830
BCL7B	683 ± 80	675 ± 107	P = 0.923

BCL7C	213 ± 67	252 ± 54	P = 0.482
BCL9	341 ± 113	355 ± 39	P = 0.854
BCL9L	2224 ± 1137	2981 ± 1104	P = 0.455
BCLAF1	4273 ± 755	5018 ± 601	P = 0.252
BCLW, BCL2L2	236 ± 114	263 ± 19	P = 0.700
BCLXL, BCL2L1	1293 ± 520	783 ± 70	P = 0.167
BID	2722 ± 966	2601 ± 800	P = 0.875
BIK	25 ± 5	16 ± 6	P = 0.131
BIM, BCL2L11	2045 ± 295	1771 ± 703	P = 0.567
BMF	705 ± 351	1090 ± 121	P = 0.147
BOK	7.72 ± 7.29	1.56 ± 2.14	P = 0.233
MCL1	81490 ± 38202	55673 ± 20213	P = 0.359
NOXA1	73 ± 44	131 ± 52	P = 0.214
PUMA	508 ± 118	559 ± 270	P = 0.780
TMBIM1	4382 ± 981	3516 ± 837	P = 0.310
TMBIM4	3078 ± 1129	2224 ± 812	P = 0.347
TMBIM6	19610 ± 6185	15273 ± 3684	P = 0.356

Table 1: **Results from NGS for the *BCL-2*-family in alphabetical order.** Normalized counts ± SD for IBD patients with a primary non-response to AZA (column two) and patients successfully treated with AZA (third column). P values ($p > 0.05$ each, t-test)

Table 2

	wild type					**IL-10$^{-/-}$**							**IL-10$^{-/-}$ x BIM$^{-/-}$**	
	A-1211212					**A-1211212**					**ABT-737**			
mg / kg / day	vehicle	3	10	30	100	vehicle	3	10	30	100	vehicle	50	vehicle	50
n =	**5**	**3**	**5**	**5**	**5**	**8**	**5**	**7**	**5**	**6**	**3**	**3**	**5**	**4**
erythrocytes (10^{12}/L)	9.48 ± 0.43	8.61 ± 0.32	9.15 ± 0.59	9.02 ± 0.55	9.29 ± 0.25	9.31 ± 0.54	9.92 ± 0.42	9.49 ± 1.07	9.37 ± 0.80	8.95 ± 0.34	8.47 ± 0.96	8.78 ± 0.43	7.38 ± 4.04	6.61 ± 3.65
platelets (10^3/µL)	**1205 ± 251**	**1194 ± 88**	**1180 ± 462**	**920 ± 280**	**1120 ± 350**	**1201 ± 268**	**1279 ± 363**	**1260 ± 282**	**1075 ± 317**	**1209 ± 379**	**1332 ± 170**	**733 ± 73**	**n.d.**	**n.d.**
											**			
lymphocytes (%)	75.3 ± 9.1	57.3 ± 24.8	57.7 ± 9.8	35.1 ± 10.4	31.6 ± 8.5	59.5 ± 8.7	52.5 ± 17.2	37.1 ± 9.6	42.5 ± 8.0	31.8 ± 12.6	56.43 ± 6.5	30.07 ± 3.0	81.18 ± 7.5	78.80 ± 10.1
	*					*					*			
monocytes (%)	**0.72 ± 0.47**	**1.13 ± 0.38**	**1.14 ±0.29**	**1.04 ± 0.43**	**1.24 ± 0.47**	**1.66 ± 1.61**	**1.25 ± 0.45**	**1.12 ±0.46**	**1.30 ± 0.22**	**1.16 ± 0.27**	**1.83 ± 0.42**	**1.23 ± 0.55**	**8.18 ± 6.92**	**13.28 ± 8.71**
granulocytes (%)	23.4 ± 9.1	40.9 ± 25.0	40.4 ± 9.4	63.4 ± 10.3	65.8 ± 9.3	39.1 ± 8.7	43.8 ± 18.9	59.1 ±10.4	54.5 ± 7.2	56.7 ± 20.6	39.47 ± 5.90	67.30 ± 3.18	5.16 ± 4.68	3.20 ± 1.20
					*							**		
neutrophiles (%)	**20.7 ± 8.7**	**25.7 ± 12.2**	**30.1 ± 5.3**	**53.6 ± 11.6**	**55.7 ± 12.8**	**38.5 ± 7.8**	**40.7 ±19.7**	**48.2 ± 6.0**	**47.2 ± 5.1**	**44.2 ± 15.5**	**34.40 ± 5.37**	**58.27 ± 0.85**	**4.36 ± 4.70**	**2.45 ± 0.96**
					*							*		

Table 2: **Hematological analyses.** PBLs from wild type mice and mice from the *Il10*$^{-/-}$ and *Il10*$^{-/-}$ x *Bim*$^{-/-}$ model of spontaneous colitis. The platelets f remained unchanged upon A-1211212, but was significantly decreased upon ABT-737, when compared to vehicle-receiving controls, n as indicated bars = SD. Statistics for mice upon A-1211212 was performed using ANOVA on ranks (Holm-Sidak method). Statistics for mice upon ABT-737 was perf using t-test, * $p < 0.05$, ** $p < 0.01$. Black arrows with a star indicate a significant difference, grey arrows indicate a trend.

Supplementary table 1

ID	Gender (male, female)	Age (years)	Duration of disease at sampling (years)	Clinical disease activity at sampling (active, quiescent, remission, refractory)	AZA / 6 MP	Additional medication (per day, unless otherwise described)	MTWSI / Montreal classification	Physical complaints	Used for
1	male	39	8	sustained remission, colitis ulcerosa	Azathioprine, 175	Oral 5-ASA Topical 5-ASA Systemic steroids VitD Prednison 5 mg	-	Prior to medication: Abdominal pain (severe) Stool is diluted and/or blood contents Post medication: No abdominal pain No evidence of any relapse	NGS qPCR
2	male	41	10	sustained remission, colitis ulcerosa	Azathioprine, 175	5-ASA 500 Proton pump inhibitor Non-steroidal anti-inflammatory drugs	-	Prior to medication: Abdominal pain Stool with blood contents Multiple stool (10 x / day) Post medication: No abdominal pain No evidence of any relapse	NGS qPCR
3	female	31	10	sustained remission, colitis ulcerosa	PuriNethol 50 mg	Oral 5-ASA, 3000 5-ASA, 2000	-	Prior to medication: Abdominal pain (severe) due to quit smoking Stool is diluted and/or blood contents Post medication: No abdominal pain No evidence of any relapse	NGS qPCR
4	female	39	13	refractory to treatment, colitis ulcerosa	Imurek 150 mg	Systemic steroids 5-ASA	-	Abdominal pain (mild) Stool is diluted and/or blood contents	NGS qPCR
5	male	28	2	refractory to treatment, colitis ulcerosa	PuriNethol 50 mg	Ursofalk 500 mg Budenofalk 3 mg Saloflak 1.5 g	-	Stool irregularities Abdominal pain (mild)	NGS qPCR
6	male	45	2	refractory to treatment, colitis ulcerosa	Imurek 150 mg	Prednison 20 mg Calcimagon Salofalk 3 g Salofalk supp. Salofalk foam Scheriproct ointment	-	Multiple stool (10 x / day) Stool is diluted and/or blood contents Abdominal pain (mild) Perianal pain Episodes of incontinence	NGS qPCR
7	female	35	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
8	male	45	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR

9	male	31	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
10	female	28	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
11	female	44	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
12	male	26	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
13	male	45	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
14	male	28	NA	healthy control	no	no mediactaion	0	no physical complaints	PI / annexin V qPCR
15	female	41	6	sustained remission, colitis ulcerosa	Azathioprine, 150	Oral 5-ASA, 8cp/j, Infliximab, 12, Topical 5-ASA, 2000	6	ing	PI / annexin V qPCR
16	female	44	9	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Systemic steroids, 40	0	ing	qPCR
17	male	35	2	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Oral 5-ASA	3	ing	qPCR
18	female	34	22	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Oral 5-ASA, 3000, Calcium, VitD	1	ing	qPCR
19	female	35	23	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Oral 5-ASA, 3000, Calcium, VitD	1	ing	qPCR
20	female	39	27	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Topical 5-ASA, 1500, Oral 5-ASA, 1500	1	ing	qPCR
21	male	49	24	sustained remission, colitis ulcerosa	Azathioprine, 150	-	1	ing	PI / annexin V qPCR

22	male	51	26	sustained remission, colitis ulcerosa	Azathioprine, 150	-	0	ing	PI / annexin V qPCR
23	male	52	27	sustained remission, colitis ulcerosa	Azathioprine, 150 mg	Systemic steroids, 30, Topical 5-ASA, 1000	8	ing	PI / annexin V qPCR
24	male	57	31	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Cyclosporine, 100	0	ing	qPCR
25	female	31	5	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Systemic steroids, Oral 5-ASA	2	ing	qPCR
26	male	29	1	sustained remission, colitis ulcerosa	Azathioprine shortly before sampling	Systemic steroids, Magnes, Calcium, VitD	18	ing	qPCR
27	male	30	2	sustained remission, colitis ulcerosa	Azathioprine, 100	-	1	ing	PI / annexin V qPCR
28	male	40	9	sustained remission, colitis ulcerosa	AZA 175 mg	Systemic steroids, Oral 5-ASA VitD	-	Prior to medication: Abdominal pain (severe) Stool is diluted and/or blood contents Post medication: No abdominal pain No evidence of any relapse	PI / annexin V qPCR
29	female	61	27	sustained remission, colitis ulcerosa	PuriNethol	Systemic steroids, Oral 5-ASA Saloflak Infliximab	-	Prior to medication: Abdominal pain (severe) Stool is diluted and/or blood contents Post medication: No abdominal pain No evidence of any relapse	qPCR
30	male	42	11	sustained remission, colitis ulcerosa	Imurek 125 mg	Quetiapin XR Citalopram 20 mg Etodolac 600 mg Novalgin Dafalgan 3g / day Mesalazin 500 mg Valaciclovir Antabus 3 x / week Vitamin D 1 x / month	-	Prior to medication: abdominal pain (moderate) Post medication: Multiple stool (10 x / day) but no abdominal pain	qPCR

31	male	31	11	sustained remission, Crohn's disease	AZA 100 mg	Budenosid oral 3mg	A: 1, L: 4, B: 4	Prior to medication: Abdominal pain Stool with blood contents Post medication: No evidence of any relapse Extraintestinal: arthritis	qPCR
32	female	40	22	sustained remission, colitis ulcerosa	AZA 100 mg	5ASA oral 4.5 g vitamin D corticosteroids Scheriproct sopp.	1	Prior to medication: Abdominal pain Stool is diluted and/or blood contents Post medication: Multiple stool (1 – 2 x / day)	qPCR
33	female	32	4	sustained remission, Crohn's disease	PuriNethol 50 mg	Salazopyrin 500 mg	-	Prior to medication: Abdominal pain Stool is diluted and/or blood contents Post medication: Multiple stool (5 – 6 x / day) but no abdominal pain Augmented arthralgia and cramps Rhinosinusitis	qPCR
34	female	60	> 2	sustained remission, Crohn's disease	Imurek shortly before sampling	no	-	Prior to medication: Abdominal pain Perianal fistula Post medication: No evidence of any relapse	qPCR
35	female	42	27	sustained remission, colitis ulcerosa	Imurek 150 mg	Budenofalk 3 mg Saloflak 1.5 g	-	Prior to medication: Abdominal pain Post medication: No evidence of any relapse No abdominal pain	qPCR
36	male	29	2	sustained remission, colitis ulcerosa	Purinethol 50 mg	Entyvio Ursofalk 500 mg Budenofalk 3 mg Saloflak 1.5 g Vitamin D	-	Prior to medication: Abdominal pain Stool is diluted Post medication: Stool irregularities	qPCR
37	female	26	14	sustained remission, Crohn's disease	Purinethol 50 mg	Vi-De 3	-	Prior to medication: Abdominal pain (moderate) Stool (3 – 4 x / day) Stool is diluted Post medication: Infrequent constipation No evidence of any relapse	qPCR
38	female	38	13	sustained remission, colitis ulcerosa	Purinethol 50 mg every other day	5ASA oral 1.5 g 5ASA rectal every third day	1	Prior to medication: Abdominal pain (severe) Stool is diluted and/or blood contents Post medication: Stool (1 – 2 x / day) No abdominal pain No evidence of any relapse	qPCR

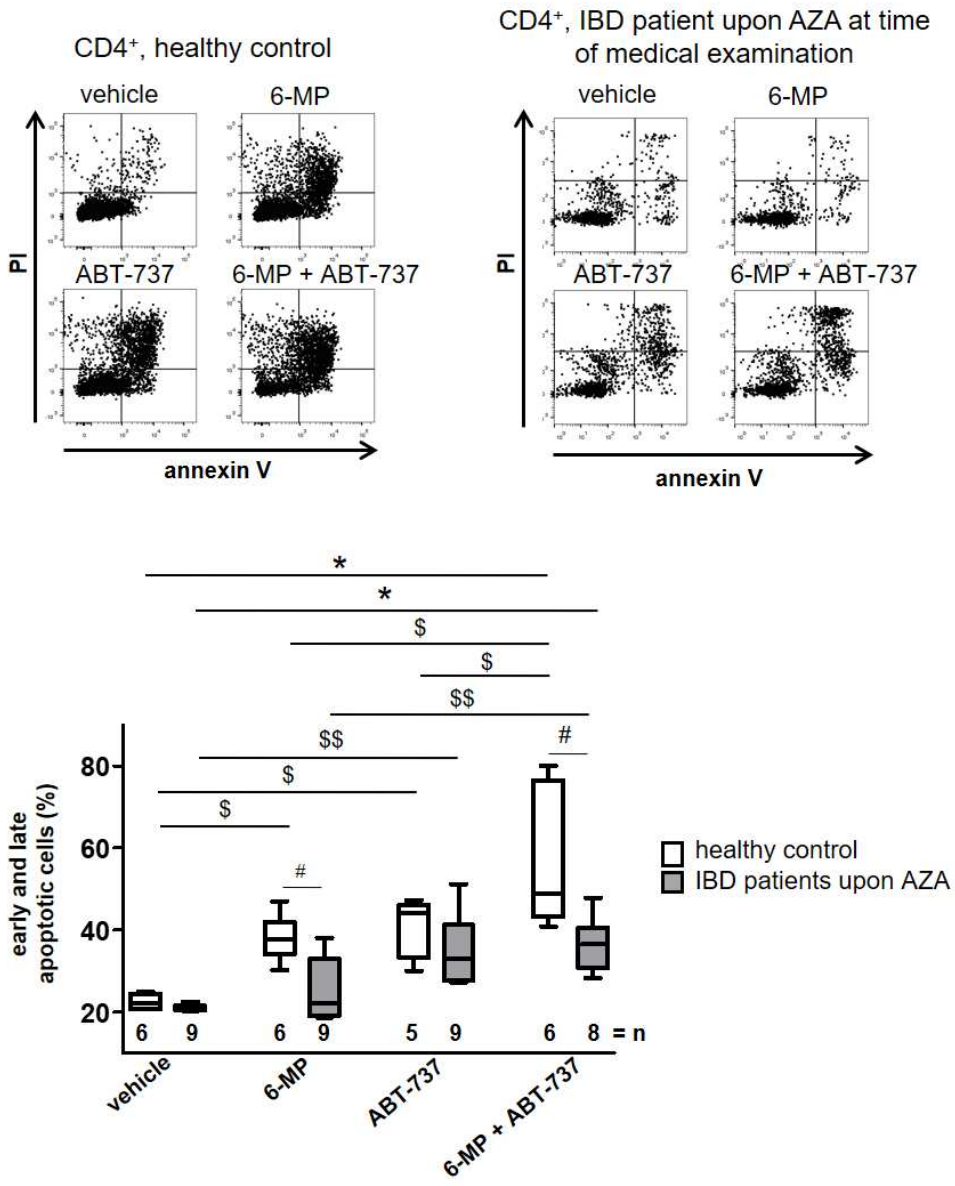
39	male	62	3	refractory to treatment	Azathioprine	Systemic steroids	6	ing	qPCR
40	male	64	6	refractory to treatment	Azathioprine	Systemic steroids, 10, Calcium VitD	8	ing	qPCR
41	female	24	1	refractory to treatment	Azathioprine	Oral 5-ASA, Topical 5-ASA, 2000	0	ing	qPCR
42	female	27	4	refractory to treatment	Azathioprine	Infliximab, 6.3	0	ing	qPCR
43	female	26	0	refractory to treatment	Azathioprine, 150	Oral 5-ASA, Calcium, VitD	2	ing	qPCR
44	female	27	1	refractory to treatment	Azathioprine	Infliximab, 5.1, , Calcium, VitD	3	ing	qPCR
45	female	28	2	refractory to treatment	Azathioprine	Oral 5-ASA, 4000, Infliximab, 5, Magnes, Calcium	8	ing	qPCR
46	male	39	4	refractory to treatment	Azathioprine	Topical 5-ASA, Oral 5-ASA	7	ing	qPCR
47	male	40	5	refractory to treatment	Azathioprine	Oral 5-ASA, 4000, Topical 5-ASA, 4000, Mutaflor	6	ing	qPCR
48	male	41	6	refractory to treatment	Azathioprine	Oral 5-ASA, 4000, Topical 5-ASA, 4000	3	ing	qPCR
49	male	42	8	refractory to treatment	Azathioprine	Oral 5-ASA, 4000	7	ing	qPCR
50	female	29	9	refractory to treatment	Azathioprine	No	-	ing	qPCR
51	male	41	12	refractory to treatment	Azathioprine	No	-	ing	qPCR
52	male	60	5	refractory to treatment, Crohn's disease	Purinethol 50 mg	Budenofalk foam Salofalk sup. Mutaflor Alendronat 70 mg / week Dancor 10 mg po, Amiodarone 200 mg po, Nebivolol 5 mg po, Pravalotin 20 mg po	-	Abdominal pain (mild but increasing) Stool (5 x / day), blood contents Calprotectin 770 µg / g stool	qPCR
53	male	28	5	refractory to treatment, colitis ulcerosa	Imurek 50 mg	Salofalk 1.5 g Budenofalk foam Salofalk supp. 1g	6	Multiple stool (5 – 6 x / day) Abdominal pain (mild)	PI / annexin V qPCR

54	male	45	2	refractory to treatment, colitis ulcerosa	Imurek 150 mg	Prednison 20 mg Calcimagon Salofalk 3 g Salofalk supp. Salofalk foam Scheriproct ointment	-	Multiple stool (10 x / day) Stool is diluted and/or blood contents Abdominal pain (mild) Perianal pain Episodes of incontinence	PI / annexin V qPCR
55	male	30	4	refractory to treatment, Crohn's disease	Imurek 50 mg	No	A:1, L:1, B:1	Watery diarrhea Multiple stool (2 – 10 x / day)	PI / annexin V qPCR
56	female	40	22	refractory to treatment, colitis ulcerosa	AZA 100 mg	5ASA oral 4.5 g vitamin D corticosteroids	6	Evacuation of mucus since two weeks Multiple stool Lower abdominal pain (moderate)	qPCR

MTX = methotrexat, CS = cyclosporine, NA = not applicable, ing = information not interrogated

Supplementary table 1: Characteristics of healthy controls and patients with IBD

Figure 1 A


CD4+, healthy control
vehicle
6-MP
ABT-737
6-MP + ABT-737
PI
annexin V
CD4+, IBD patient upon AZA at time of medical examination
vehicle
6-MP
ABT-737
6-MP + ABT-737
PI
annexin V
early and late apoptotic cells (%)
healthy control
IBD patients upon AZA
6 9 6 9 5 9 6 8 = n
vehicle
6-MP
ABT-737
6-MP + ABT-737

B

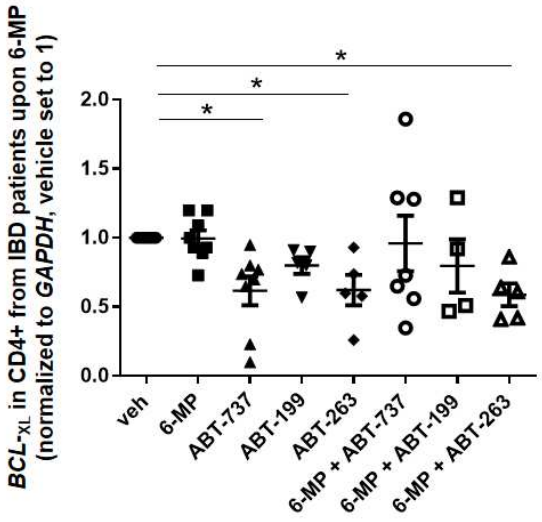
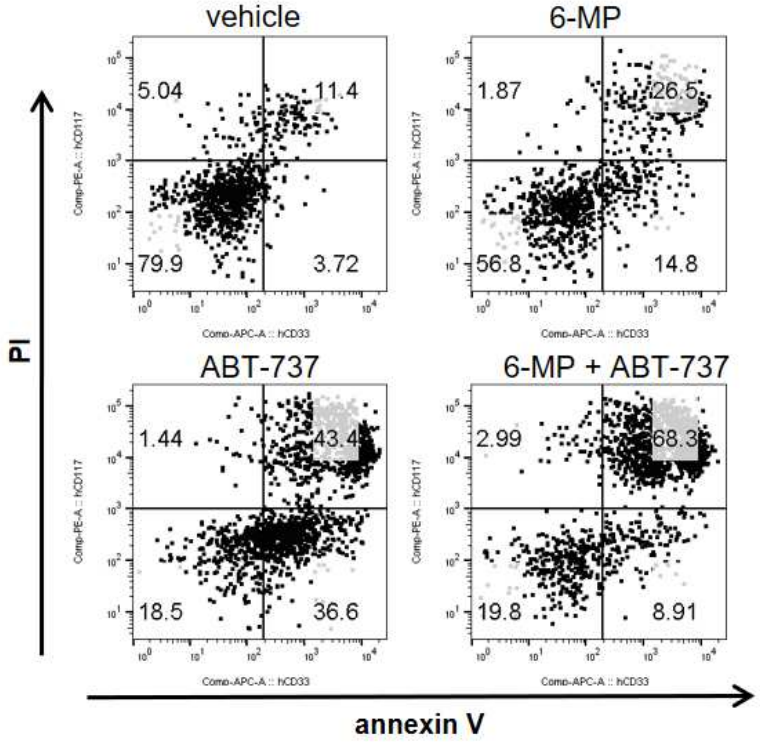

BCL-XL in CD4+ from IBD patients upon 6-MP (normalized to GAPDH, vehicle set to 1)
veh
6-MP
ABT-737
ABT-199
ABT-263
6-MP + ABT-737
6-MP + ABT-199
6-MP + ABT-263

Figure 2

Dot plot: CD4⁺, primary non-responder IBD patient upon AZA (patient #53)



CD4⁺, three primary non-responder IBD patients upon AZA (patients #53, 54, 55)

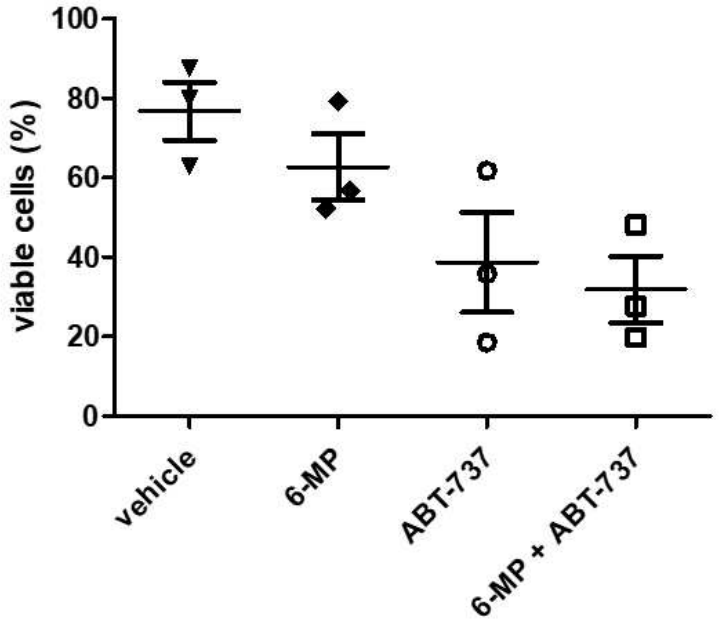


Figure 3

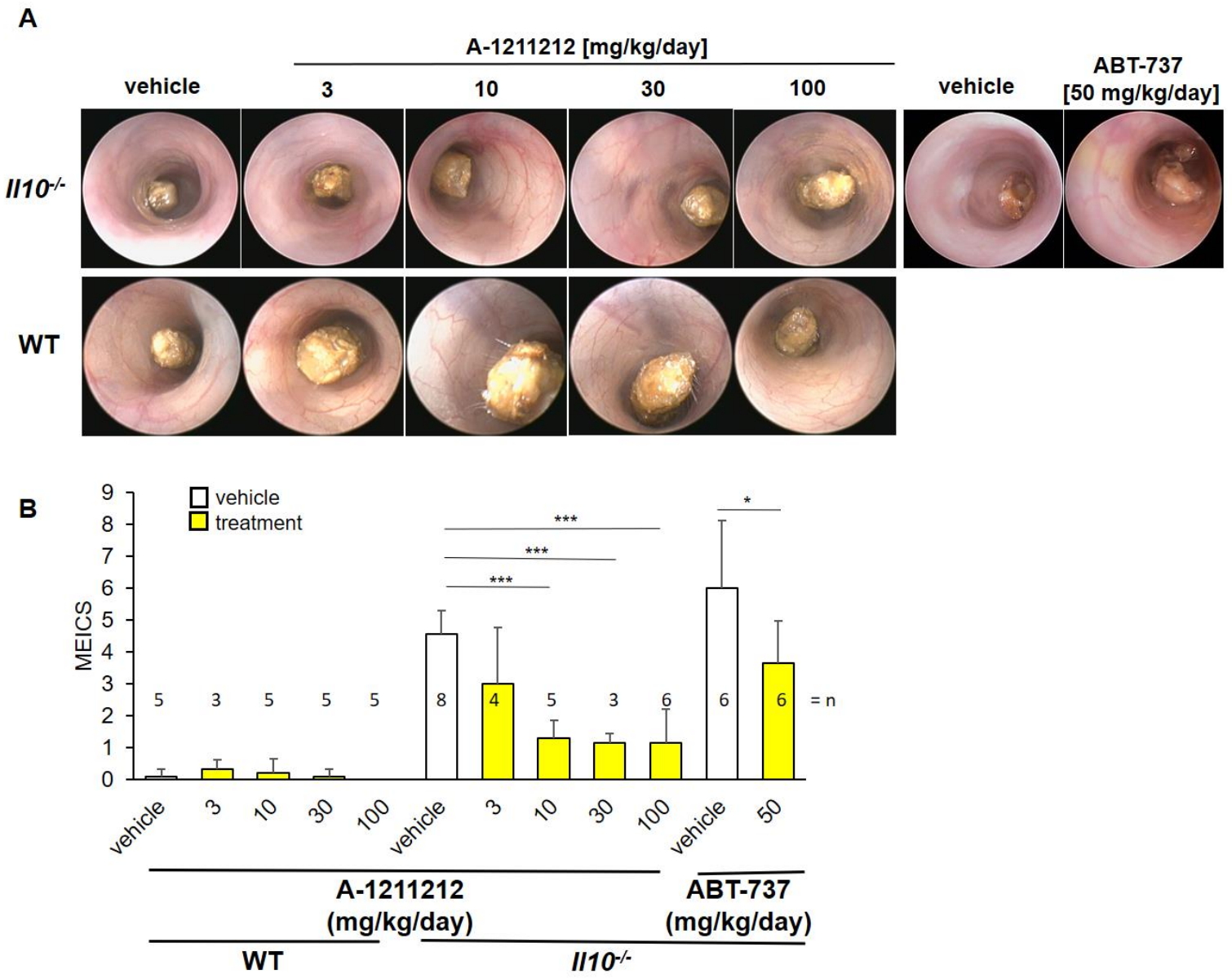

A
A-1211212 [mg/kg/day]
vehicle
3
10
30
100
vehicle
ABT-737
[50 mg/kg/day]
Il10-/-
WT
B
vehicle
treatment
MEICS
***
***
***
*
5 3 5 5 5 8 4 5 3 6 6 6 = n
vehicle 3 10 30 100 vehicle 3 10 30 100 vehicle 50
A-1211212
(mg/kg/day)
ABT-737
(mg/kg/day)
WT
Il10-/-

Figure 4

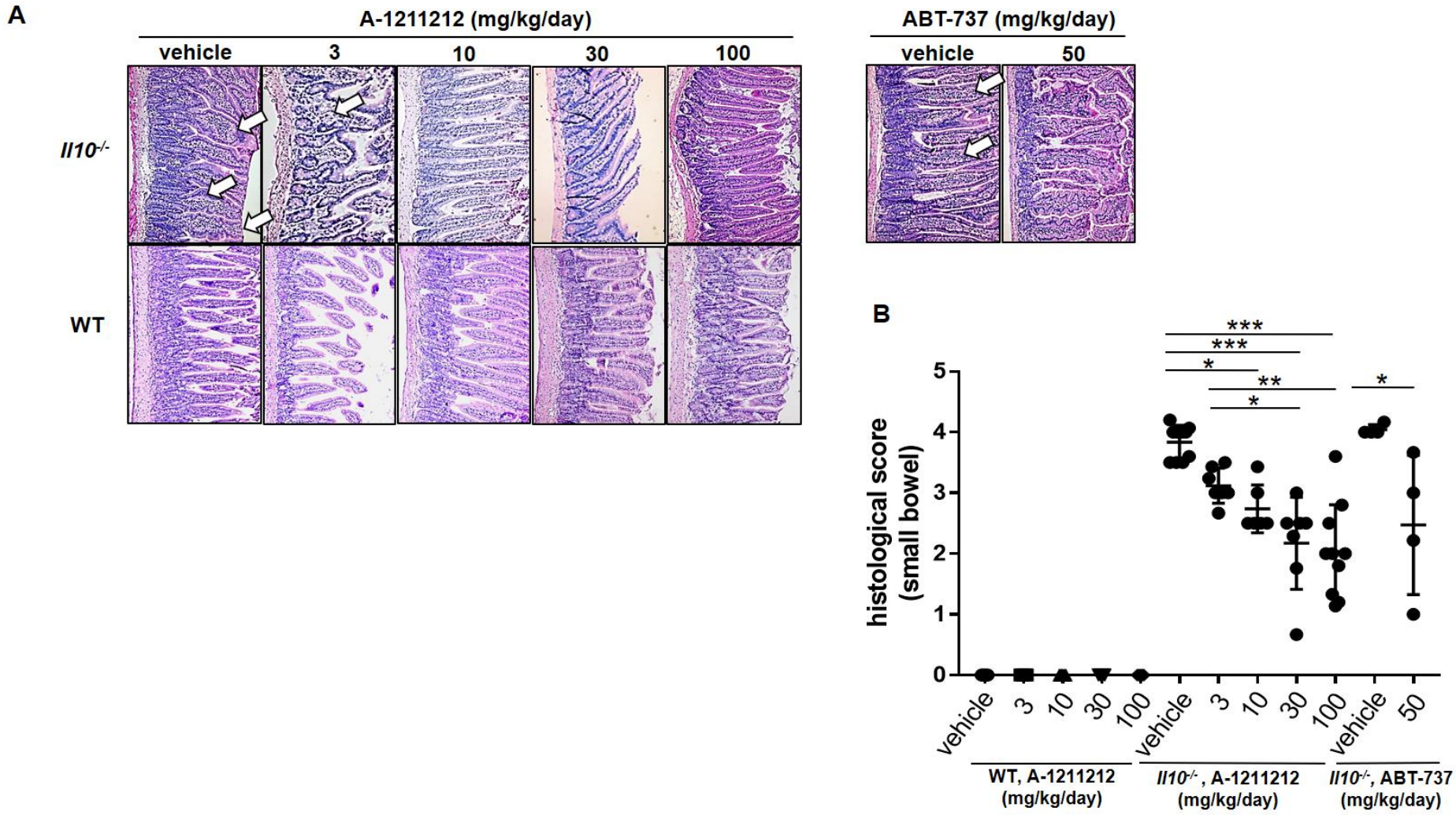

A
A-1211212 (mg/kg/day)
vehicle
3
10
30
100
ABT-737 (mg/kg/day)
vehicle
50
Il10-/-
WT
B
histological score (small bowel)
0
1
2
3
4
5
***
***
*
**
*
*
vehicle
3
10
30
100
vehicle
3
10
30
100
vehicle
50
WT, A-1211212 (mg/kg/day)
Il10-/-, A-1211212 (mg/kg/day)
Il10-/-, ABT-737 (mg/kg/day)

Figure 5

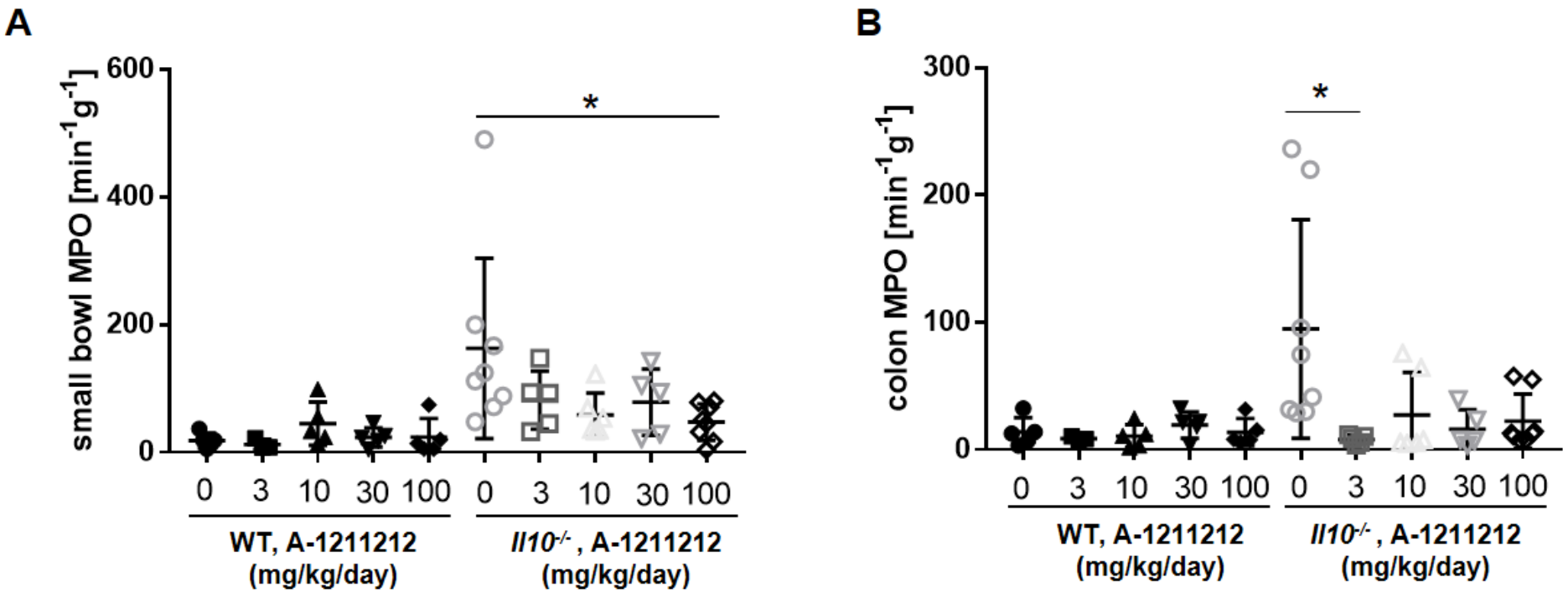

A
small bowl MPO [min-1g-1]
600
400
200
0
*
0 3 10 30 100 0 3 10 30 100
WT, A-1211212 (mg/kg/day)
Il10-/- , A-1211212 (mg/kg/day)
B
colon MPO [min-1g-1]
300
200
100
0
*
0 3 10 30 100 0 3 10 30 100
WT, A-1211212 (mg/kg/day)
Il10-/- , A-1211212 (mg/kg/day)

Figure 6

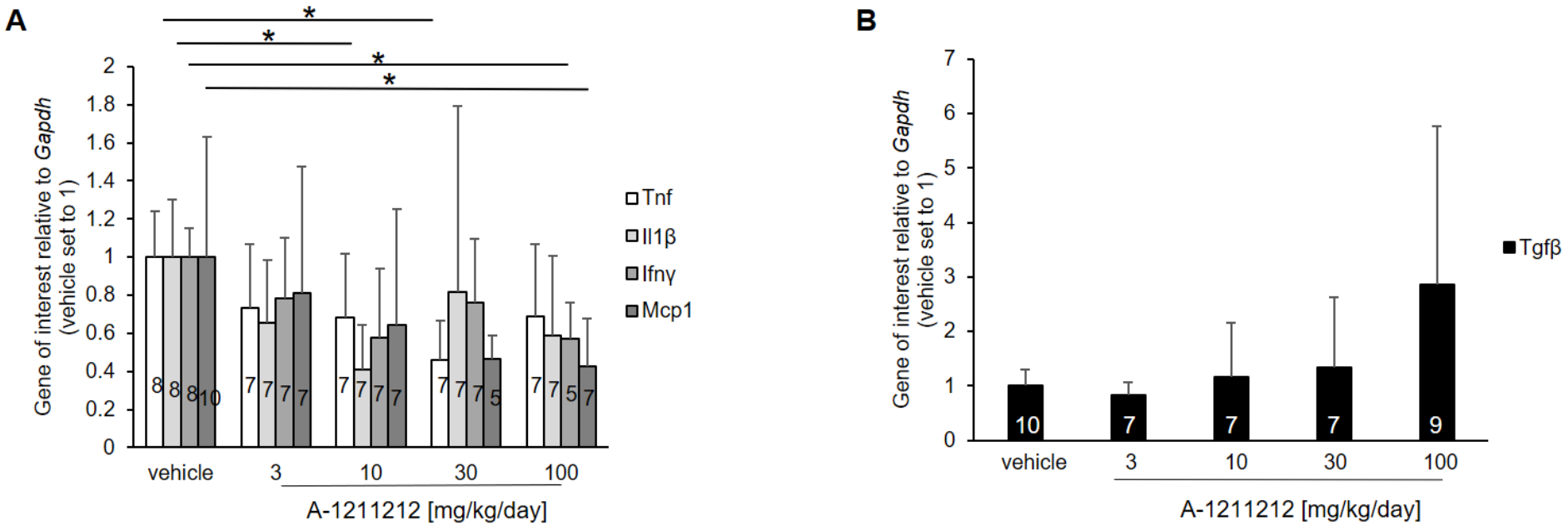
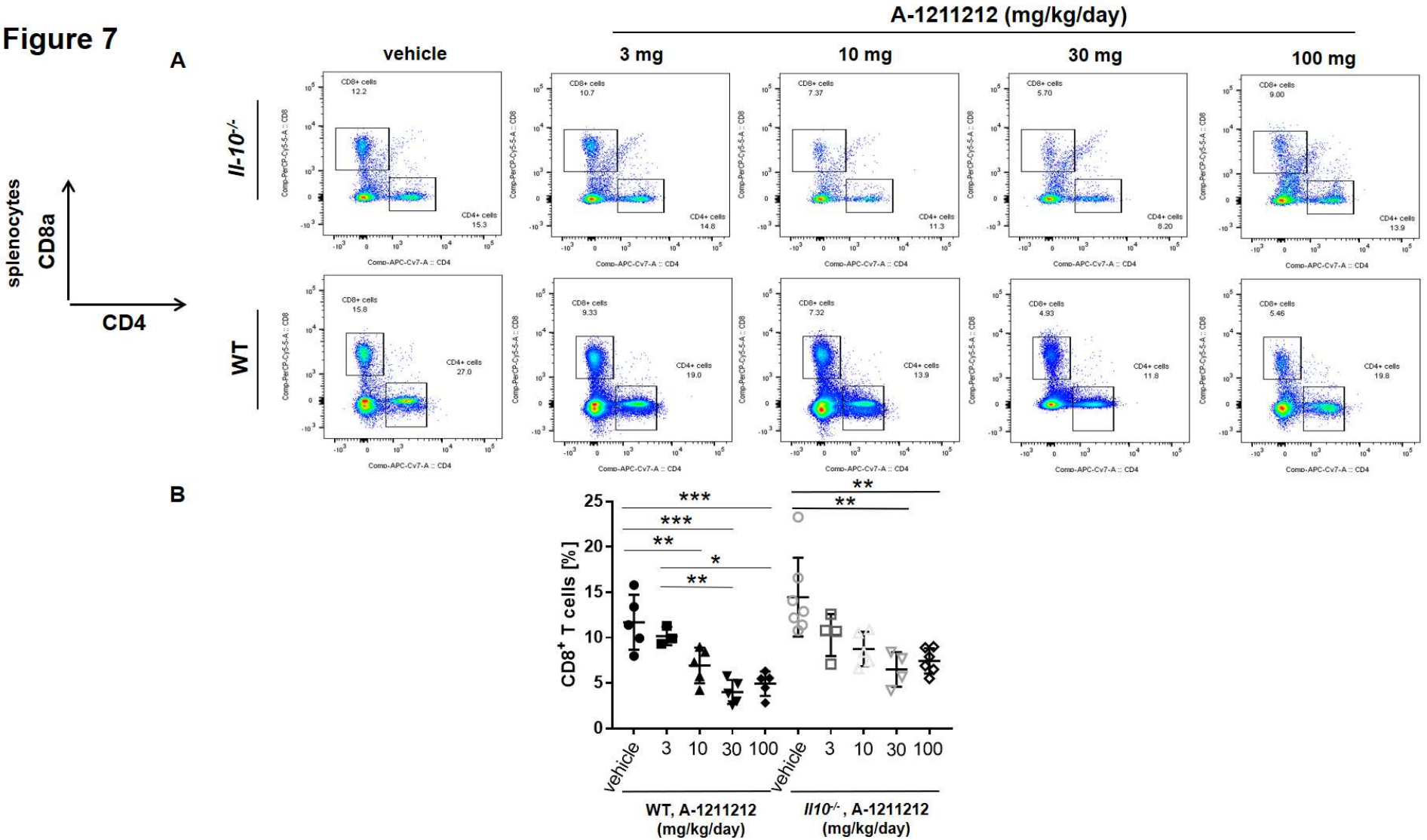
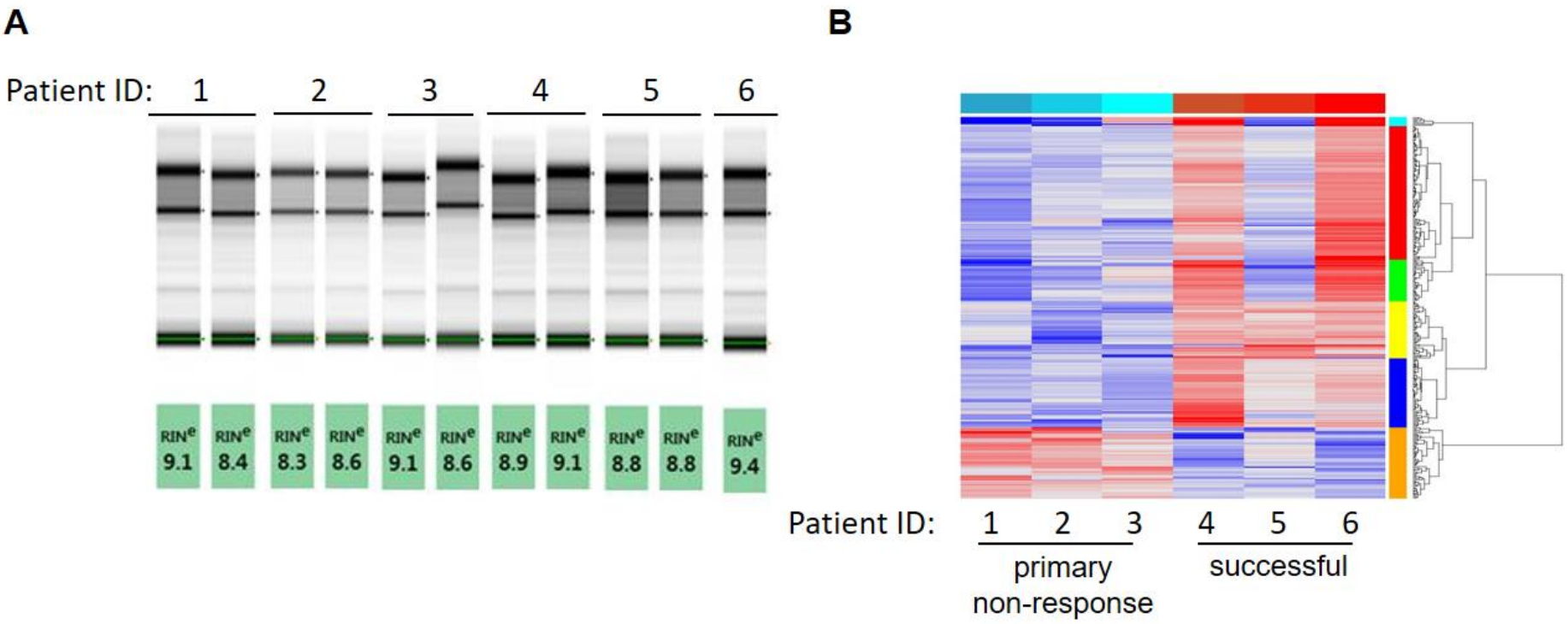

A
B
Gene of interest relative to Gapdh (vehicle set to 1)
A-1211212 [mg/kg/day]
vehicle
3
10
30
100
Tnf
Il1β
Ifnγ
Mcp1
Tgfβ
*

Figure 7

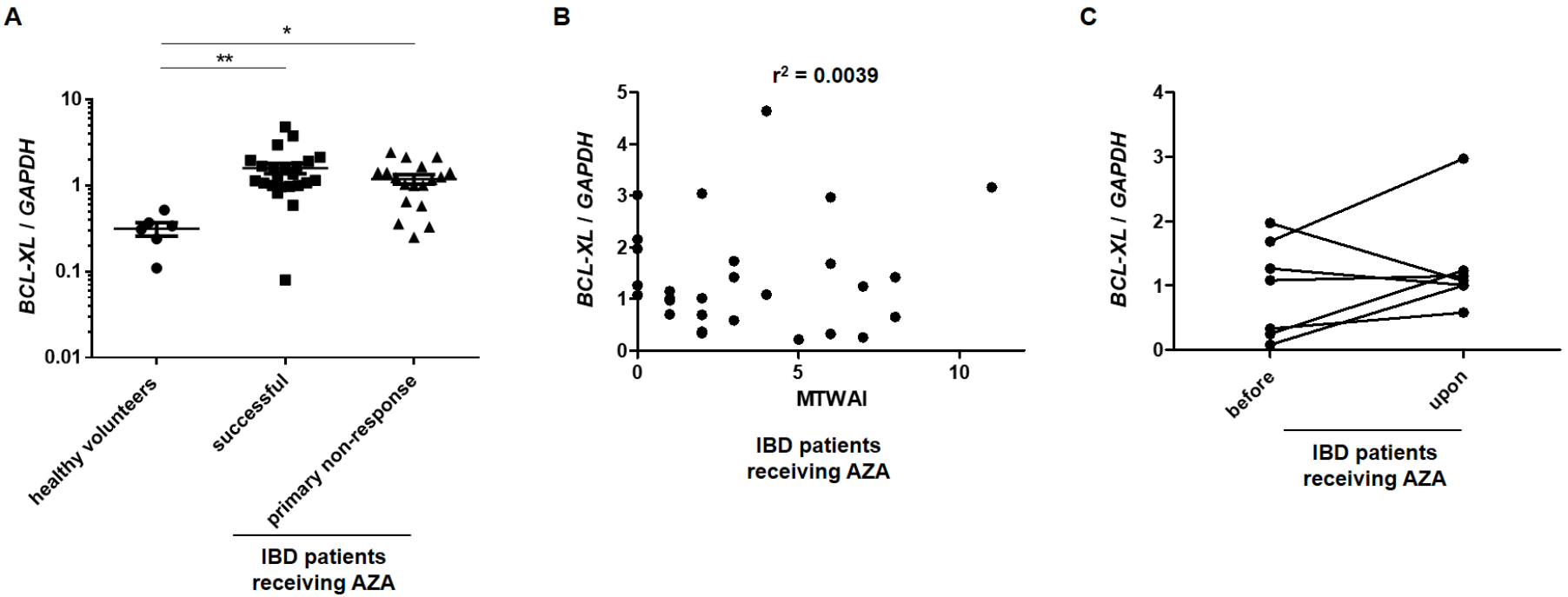

A
A-1211212 (mg/kg/day)
vehicle
3 mg
10 mg
30 mg
100 mg
Il-10-/-
WT
splenocytes
CD8a
CD4
B
CD8+ T cells [%]
vehicle
3
10
30
100
WT, A-1211212 (mg/kg/day)
Il10-/-, A-1211212 (mg/kg/day)

Supplementary figure 1



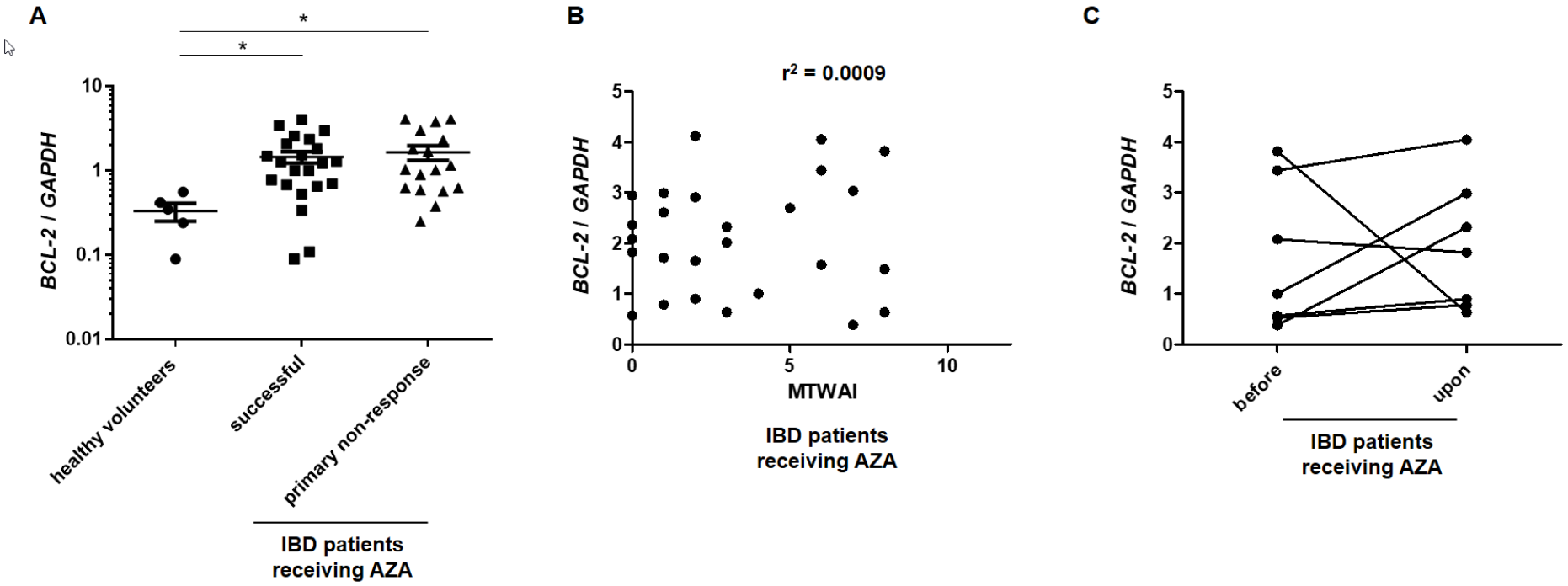
Supplementary figure 1: **mRNA expression of members from the *BCL-2*-family are not useful as prognostic factors for the success of AZA therapy.** NGS from patients successfully treated with AZA and patients showing a primary non-response upon AZA, three each. (A) Tapestation quality control of mRNA from six patients selected for NGS. Following erythrocyte lysis and RNA isolation with the miRNeasy kit from 3 ml whole blood, all patients mRNA selected showed a RIN > 8.3. (B) Following mRNA sequencing patients are clustered in two subgroups.

Supplementary figure 2



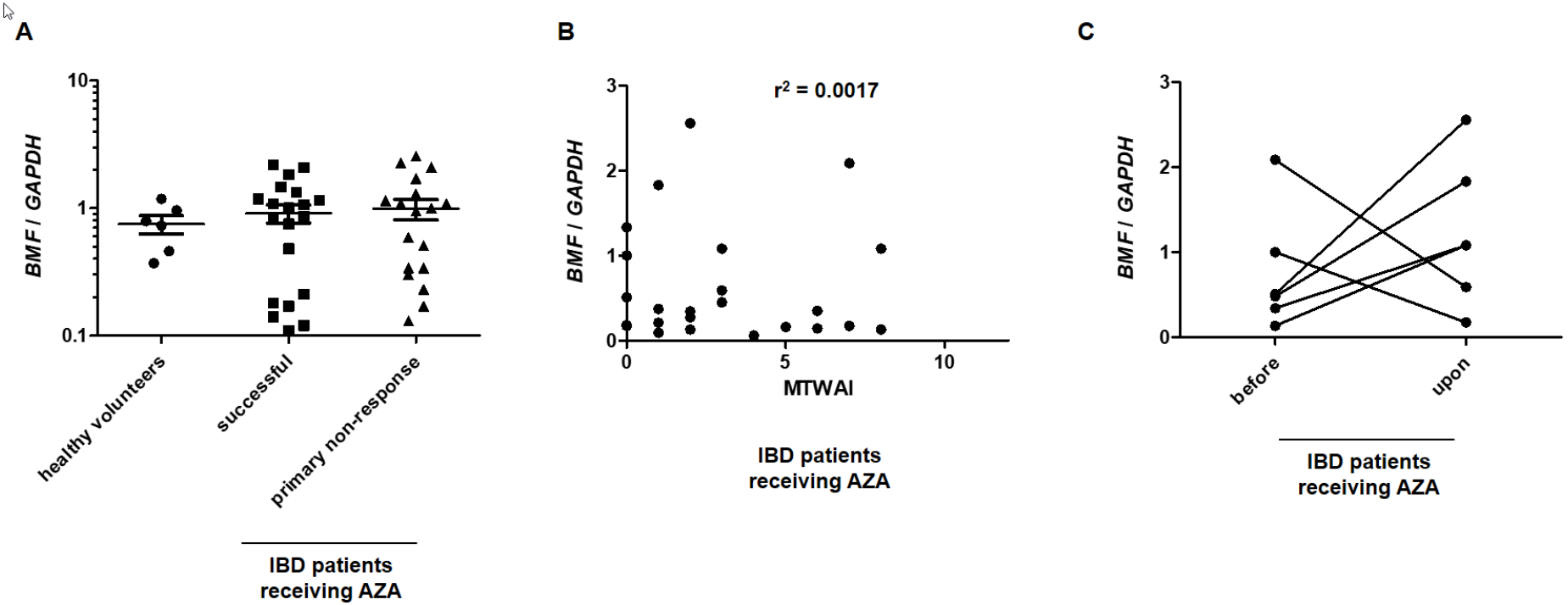
Supplementary figure 2: ***BCL-XL* is not a prognostic factor for the success of AZA therapy.** qPCR from buffy coat isolated from healthy volunteers and IBD patients. (A) *BCL-XL* showed no difference of successfully treated IBD patients compared to patients with a primary non-response (error bars = SD, * $p < 0.05$, ** $p < 0.01$, ANOVA, Dunn's multiple comparison test). (B) No significant correlation between *BCL-XL* and MTWAI ($r^2 = 0.0039$). (C) Time course of *BCL-XL* mRNA expression in IBD patients before treatment and upon AZA.

Supplementary figure 3



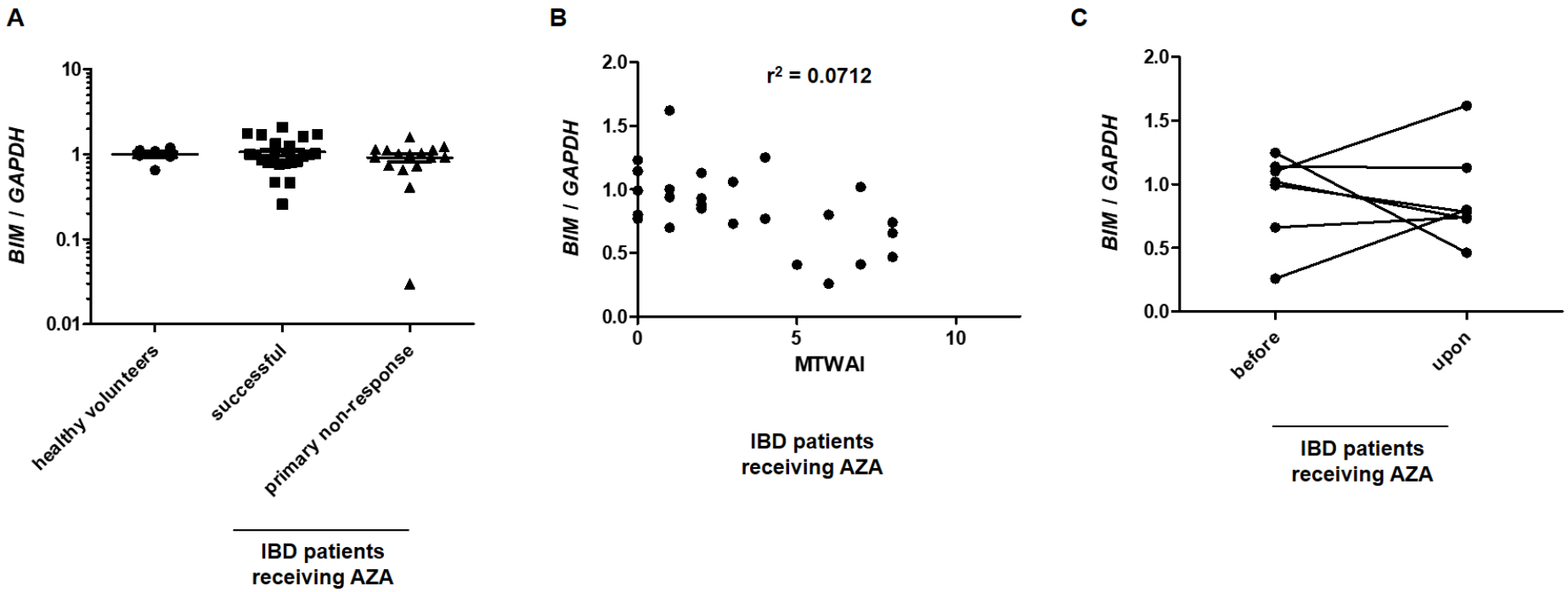
Supplementary figure 3: ***BCL-2* is not a prognostic factor for the success of AZA therapy.** qPCR from buffy coat isolated from healthy volunteers and IBD patients. (A) *BCL-2* showed no difference of successfully treated IBD patients compared to patients with a primary non-response (error bars = SD, * $p < 0.05$, ANOVA, Dunn's multiple comparison test). (B) No significant correlation between *BCL-2* and MTWAI ($r^2 = 0.0009$). (C) Time course of *BCL-2* mRNA expression in IBD patients before treatment and upon AZA.

Supplementary figure 4



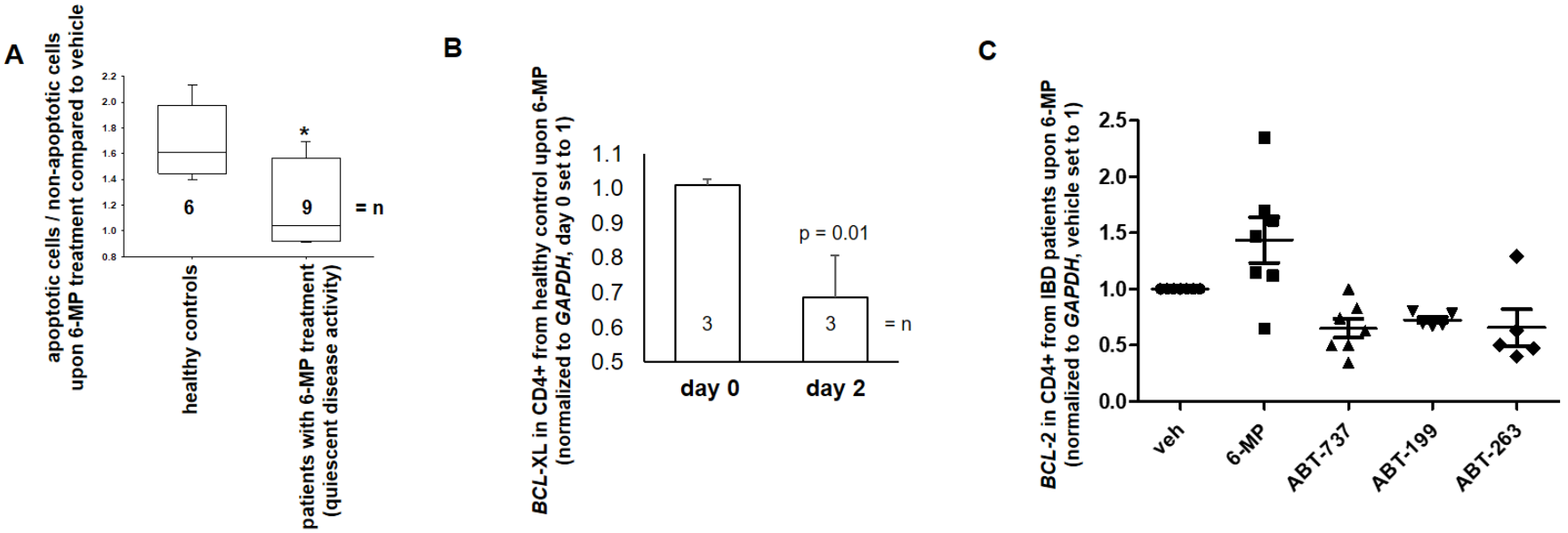
Supplementary figure 4: ***BMF* is not a prognostic factor for the success of AZA therapy.** qPCR from buffy coat isolated from healthy volunteers and IBD patients. (A) *BMF* showed no difference of successfully treated IBD patients compared to patients with a primary non-response (error bars = SD, ANOVA, Dunn's multiple comparison test). (B) No significant correlation between *BMF* and MTWAI (r^2 = 0.0017). (C) Time course of *BMF* mRNA expression in IBD patients before treatment and upon AZA.

Supplementary figure 5



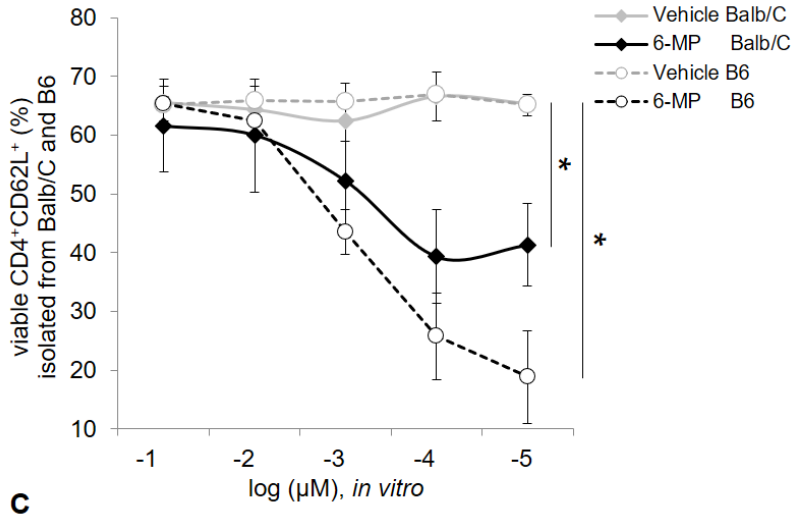
Supplementary figure 5: ***BIM* is not a prognostic factor for the success of AZA therapy.** qPCR from buffy coat isolated from healthy volunteers and IBD patients. (A) *BIM* showed no difference of successfully treated IBD patients compared to patients with a primary non-response (error bars = SD, ANOVA, Dunn's multiple comparison test). (B) No significant correlation between *BIM* and MTWAI (r^2 = 0.0712). (C) Time course of *BIM* mRNA expression in IBD patients before treatment and upon AZA.

Supplementary figure 6

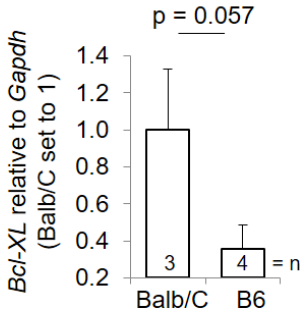


Supplementary figure 6: **BCL-2 inhibitor initiates cell death in CD4⁺ T cells from patients refractory to AZA.** Flow cytometry and qPCR. (A) Flow cytometry showing apoptosis in CD4+ isolated from PBLs from healthy donors and IBD patients (error bars = SD, * $p < 0.01$, t-test, n as indicated). (B) qPCR. *BCL-XL* in CD4⁺ from healthy donors upon 6-MP for 48 hours (error bars = SD, * $p < 0.01$, t-test, n as indicated). (C) qPCR. *BCL-2* in CD4⁺ from IBD patients upon 6-MP and pro-apoptotic small molecules (error bars = SD, ANOVA, Dunn's multiple comparison test, not significant).

Supplementary figure 7A

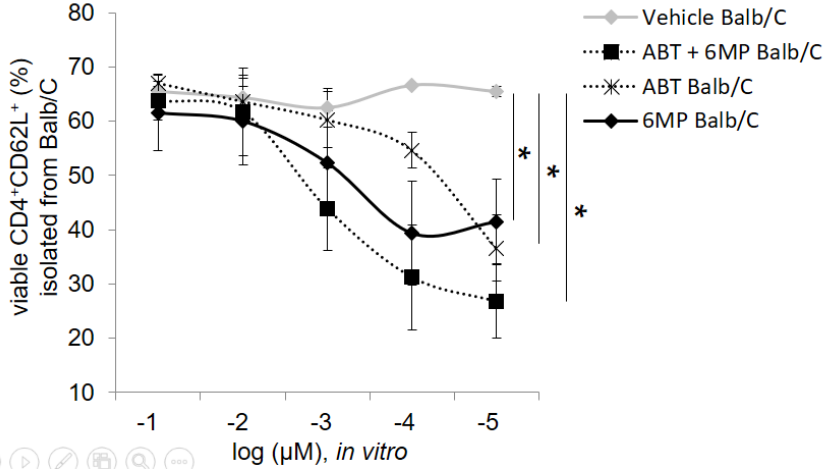


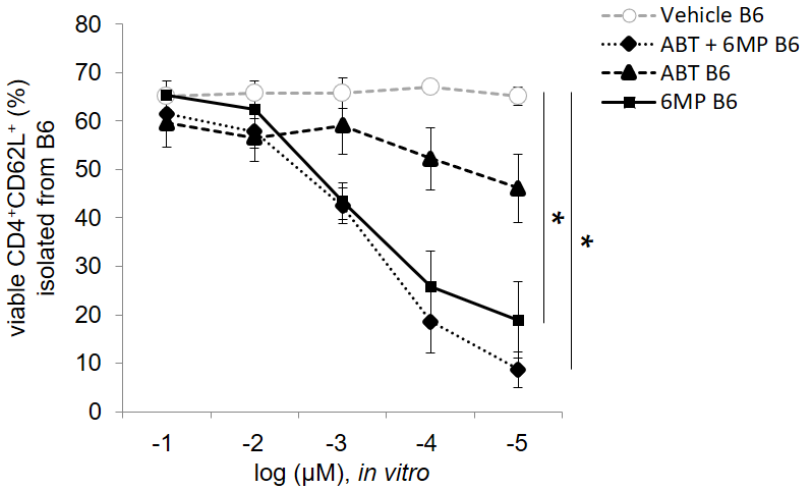
B



Supplementary figure 7: **Increased cell death in CD4⁺CD62L⁺ splencytes upon co-stimualtion with 6-MP and ABT-737.** Flow cytometry and qPCR. (A) Quantification of viable CD4+CD62L+ murine splenocytes from Balb/C and B6 upon 6-MP by flow cytometry. Error bars = SEM, * $p < 0.05$, ANOVA. (B) *Bcl-XL* mRNA expression in CD4⁺CD62L⁺ splenocytes from B6 compared to Balb/C, error bars = SEM, t-test. (C) Quantification of viable CD4+CD62L+ splenocytes isolated from Balb/C (left) and B6 mice (right) upon 6-MP, ABT-737 and co-stimulation. Error bars = SEM, * $p < 0.05$, ANOVA.

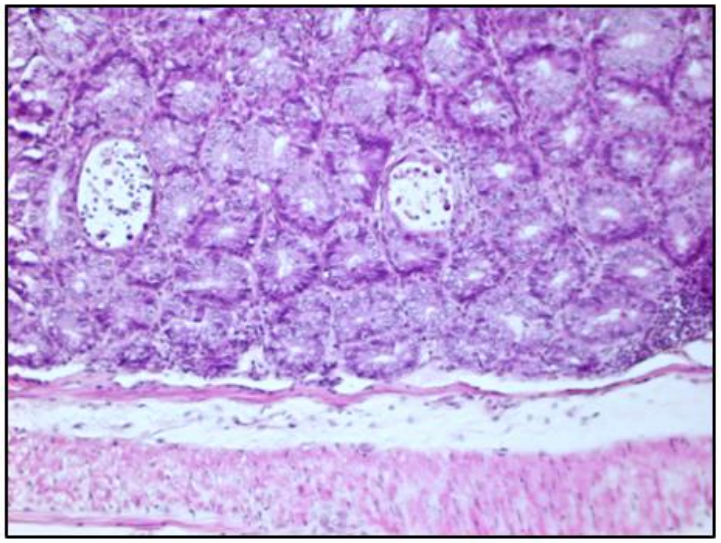
C



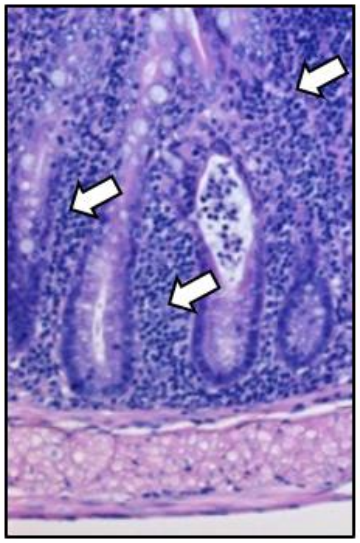


Supplementary figure 8:

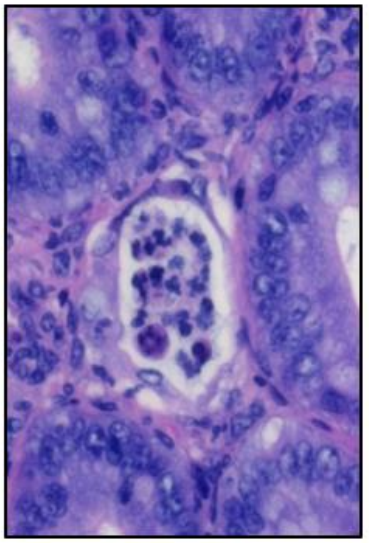
cryptitis magnification 10x



cryptitis magnification 10x

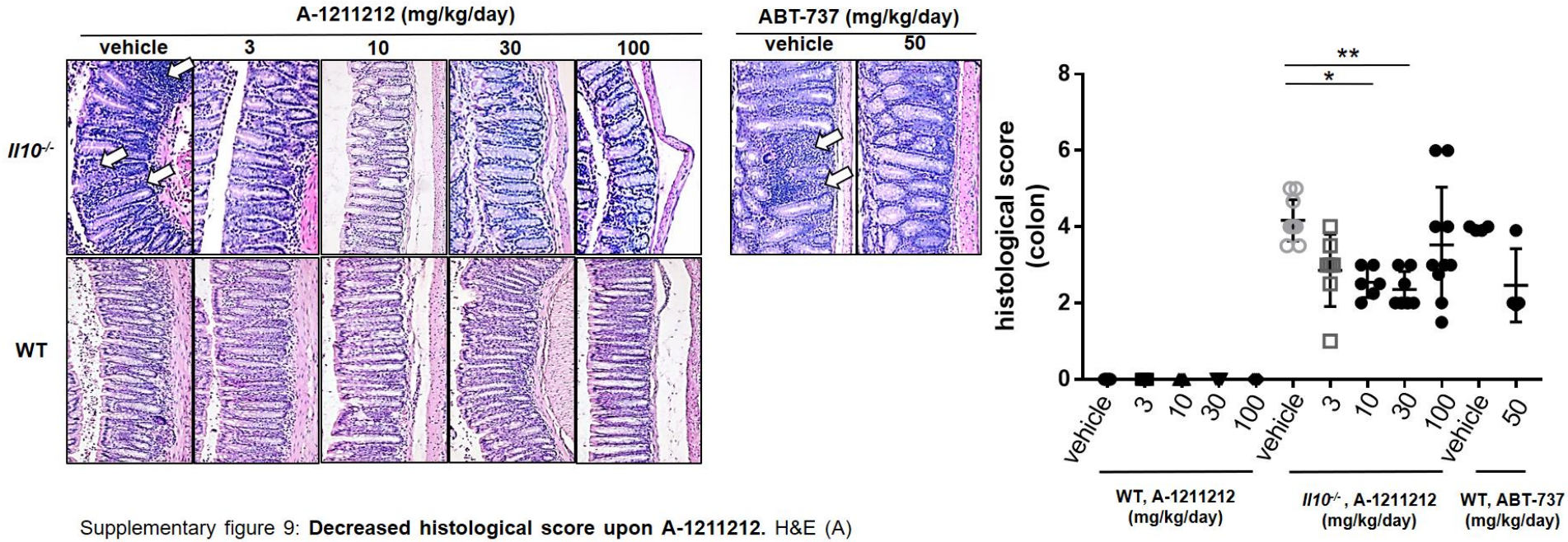


cryptitis magnification 40x



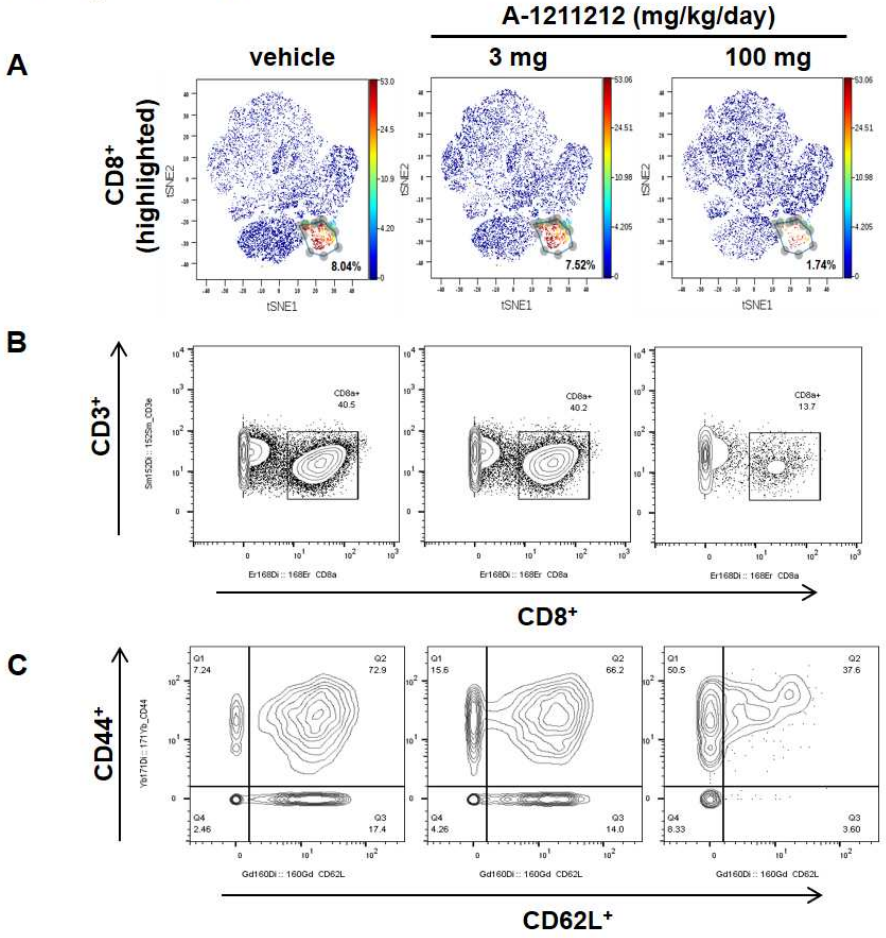
Supplementary figure 8: **Cryptitis and influx of lymphocytes.** H&E shows cryptitis in intestinal mucosa at day 0 in *Il10*$^{-/-}$ mice suffering from spontaneous colitis. Arrows = influx of lymphocytes.

Supplementary figure 9



Supplementary figure 9: **Decreased histological score upon A-1211212.** H&E (A) and histological score (B) upon both A-1211212 and ABT-737 in colon from *Il-10*$^{-/-}$ mice suffering from spontaneous colitis following 14 days of A-1211212 treatment, n as indicated, error bars = SD. Arrows = influx of lymphocytes. * $p < 0.05$, ** $p < 0.01$, ANOVA, Dunn's multiple comparison test.

Supplementary data 10



Supplementary figure 10: **CyTOF confirms decrease in CD8⁺ T cells in PBL upon A-1211212 in spontaneous colitis.**

CyTOF was performed once for each stimulation to support data from flow cytometry shown in figure 7.

(A) viSNE map showing CD8⁺ T cells encircled. Cells without a nucleus removed, doublets discrimination performed, viable and dead cells included, 10.000 cells shown each.
(B) Dot plot showing CD8⁺ T cells in the box. Cells without a nucleus removed, doublets discrimination performed, viable and dead cells included. Cells shown were gated to CD45⁺, CD19⁻, B220⁻, TCRb⁺ T cells.
(C) Contour plot showing CD8⁺ central memory T cells in the upper right corner. Cells without a nucleus removed, doublets discrimination performed, viable and dead cells included. Cells were gated to CD45⁺, CD19⁻, B220⁻, TCRb⁺ CD3⁺ CD8⁺ T cells.